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(54) **MAMMALIAN PROTEINS THAT BIND TO FKBP12 IN A RAPAMYCIN-DEPENDENT FASHION**

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(52) **U.S. Cl.** **530/413**; 530/413; 435/69.1
(58) **Field of Search** 530/413, 350; 435/69.1

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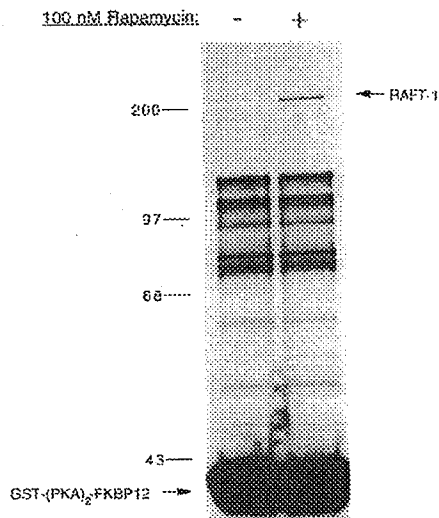* cited by examiner

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(57)   **ABSTRACT**

A protein complex containing 245 kDa and 35 kDa components, designated RAFT1 and RAFT2 (for Rapamycin And FKBP12 Target) interacts with FKBP12 in a rapamycin-dependent manner. This interaction has the pharmacological characteristics expected from the observed in vivo effects of rapamycin: it occurs at low nanomolar concentrations of rapamycin and is competed by excess FK506. Sequences (330 amino acids total) of tryptic peptides derived from the affinity purified 245 kDa RAFT1 reveals striking homologies to the predicted products of the yeast TOR genes, which were originally identified by mutations that confer rapamycin resistance in yeast. A RAFT1 cDNA was obtained and found to encode a 289 kDa protein (2550 amino acids) that is 43% and 39% identical to TOR2 and TOR1, respectively.
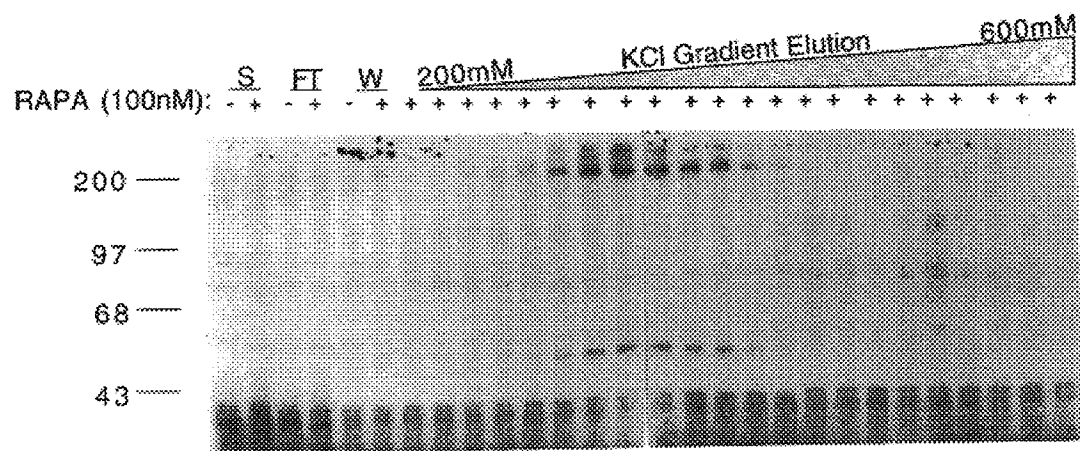
2 Claims, 10 Drawing Sheets

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FIG. 1

FIG. 2A

FIG. 2B

A3040

FIG. 3

FIG. 4A

(Protein sequence alignment of RAFT1, TOR2, and TOR1)
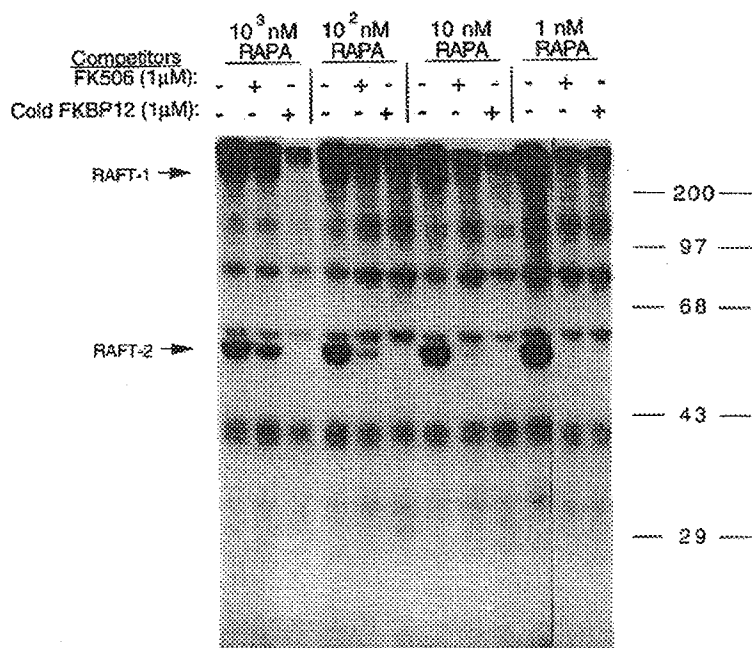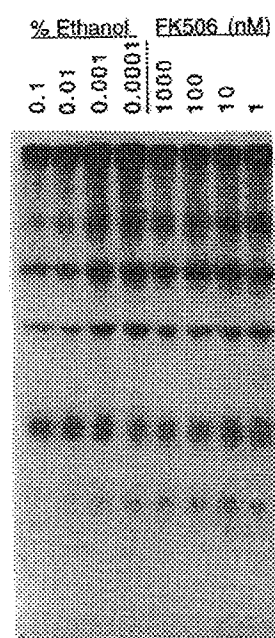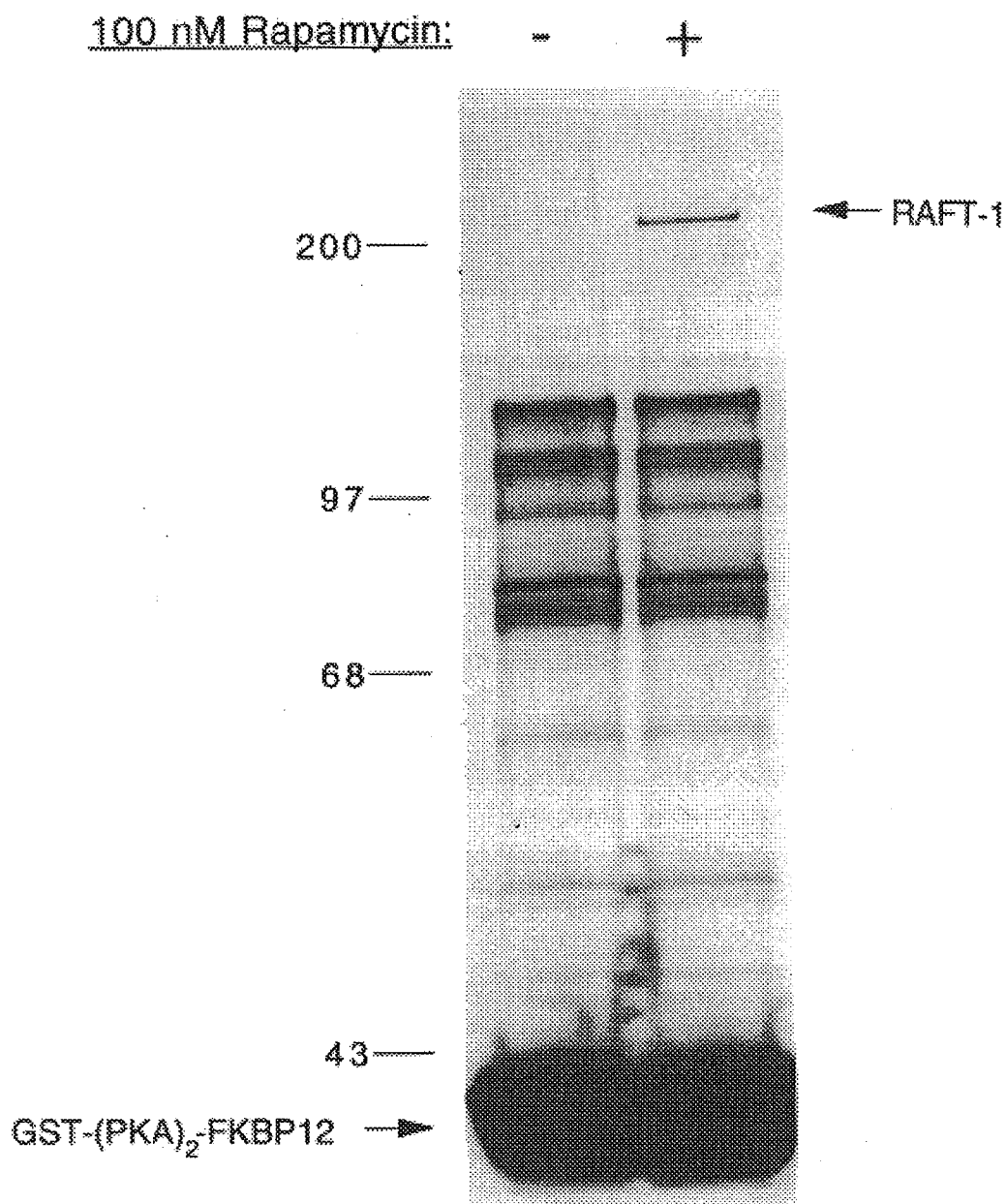
RAFT1 MLGTGPATATAGAATSSNVSVLQQFASGLKSRNEETRAKAAKELQHYVTME
TOR2   SAGHIGKISFVDSELDTTFSTLNLIFDKLKSDVPQERASGANELSTTLTSL
TOR1   TSSRFDGVVIGSNGDVNFKPIEEKIFRELTSDYKEERKLASISLFDLLVSL

RAFT1 STRIGRFANMERNLLPSSDPVMEMASKAIGREAMAGDTFTAEYVEREVKR
TOR2   QT--SREANXERVEIPSSDIEVMRLAANTLGRETVPGGTLTSDFVEREVRT
TOR1   ET--SRLAGYERGEIPSNDVEVMRLAAKTLGKEAVPGGTYTSDFVEREIKS

RAFT1 AVWDPKQAEREGAVAALRACLILTTQREPKEMQKPQWYRHTEEEAEKGFDE
TOR2   PLRDAKLITRLDAAVALGKCETIIQDRDPA--LGKQWFQRLEQGCTHGLS-
TOR1   ALRDPHLVERIDASITEAKCESTLRNRDPQ--LTSQWVQRLATSCEYGFQ-

RAFT1 DLMGFGTKPKRHITPFTSFQAVQPQQSNALVGLLGYSSHQGLMGFGASPSPT
TOR2   ----
TOR1   ----

RAFT1 ETDTQYLQDTMNHVLSCVKKEKER------------TAAFQALGL
TOR2   FTK-KYEDRIMVHYER-------YLKNIDMNAANSDKPFILVSIGD
TOR1   EAG-KYEHQIMDNYEEILTNAPAKKIPHLKD------DKPOILISIGD

RAFT1 GRGIOODI-KELEEPMEAVGLSPALTAVLYDESROIPQLKKDIODGELKME
TOR2   GPAFAKHLNKDEENLMENCPMSDHMQETLMILNEKIPSLESTVNSRIENLE
TOR1   GPVLGKLLNRNIEDLMFKCPLSDYMQETFQIETERIPSLGPKINDEEENLV

RAFT1 SDVASETLALRTLGSFEFEGHSLTQFEVRHCADHFLNSEHKEIRMEAARTCS
TOR2   TDAOILIOCFKMLQLIHHQ-YSLTEFVRLITISYIEHEDSSVRKLAALTSC
TOR1   NDIKIEIOAFRMLKNIKSR-FSEVEFVRIVALSYIEHTDPRVRKLAALTSC

RAFT1 LDERFDAHLAOAENEQALFVALNDQVFETRELAETCTVGRLSSMNPAFVMPF
TOR2   LGSNFDPOLAQPDNERLEFMAENDEIFGIOLEAJKIIGRLSSVNPAYVVPS
TOR1   LNPCFDPOLAQPDNERLEFTALHDESFNIOSVAMELVGRLSSVNPAYVIPS

RAFT1 KDPDPDPNPGVINNVLATIGELAOVSGLEMRKWVDELFVIIMDMLQDSSLL
TOR2   Q-----DASSAVASTALKVLGELSVVGGKEMTRYLKELMPLIJNTFQDOSNS
TOR1   Q-----DTSSTVASTALRTIGELSVVGGEDMKIYLKDEFPLIIKTFQDOSNS

FIG. 4B

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                                                                      100
LREMSQEESTRFYDQLNHHIFELVSSSDANERKGGILAIASLIGV----EGGN              169
AREVSAEQFORFSNSLNNKIFELIHGFTSSEKIGGILAVDTLISFYLSTEELPN             157
EHELSIEEFQAISNDINNKILELVHTKKTNTRVGAVESIDTLISFYAYTERLPN

                                                                      196
ALEWL--GADRN------EGRRHAAVEVLRELAISVPTFFQQVQPFFDNIFV              271
CIDWLLTADNNS-SSSKLEYRRHAALEIIKALADNSPYLLYPYVNSILDNIWY              260
CLEWLETASTEKNSFSSSKPDHAKHAALEIITALAENCPYLLYQYLNSILDNIWR

                                                                      301
TLAKEKGMNRDDRIHGALLLNEEVRISSMEGERLREEMEEITQQQLVHDKYCK              350
--------LNTNDSVHATLEVFREELSLKA--------                             339
--------VNTLECIHASLEVYKEITLFLKD--------

                                                                      406
KSTLVESRCCRDLMEEKEDQVCQWVLKCRSSKNSLIQMTILNLLPRLVAFRPSA             384
--------PYLRDKYDDIYKSTMKYKEYKFDVIRREVYAICPLEAAEFDRAI               373
--------PFLNQVEDQMCLNCIAYENHKAKMIREKIYOIVPLEASENPQL

                                                                      493
LSVAVRSEFKVXLPRVEDIIRAAEPPKDFAHKROKTVOVDATVETCISMLARAM            472
IAFEVGSSISPXMTLIEDNIREGERTK-FKVRKO---FEKDLFYCIGKLACAL             463
IAYEVGPDIAPMVKQILDYIEHDLOTK-FKFRKK---FENEIFYCIGRLAVPL

                                                                      576
SLVLMHKPLRHRGMPK-----------------GLAHOLASPGLTTLPEA               568
SISLSGEKFIQ------SNOYDFNNOFSIEKARKSRNOSFMKKTGESN-DDI              559
CSTLSGTRFIQRGSPMEIPS-----FSRERAREWRNKSILQKTGESN-DDN

                                                                      678
RELTPSIHLISGHAHVVSOTAVOV--VADYLSKLLVVGITDPDPDIRYCVLAS            664
DEFI--------KDDICKQTSVHALHSVSEVLSKLEMIAITDPVAEIRLEIEQH            655
EIYV--------KDNICKQTSLHSLNTVSEVLSKLEAITIADPLODIRLEVLKN

                                                                      783
LRKMEIOILTELEHSGIGRIKEOSARMLGHEVSNAPRLIRPYMEPILKALILKL           769
ERKTELELLTOLKFSNMPKKKEESATLLCTEINSSDEVAKPYIDPILDVILPKC          760
IRKILELLTKLKFSTSSREKEETASLLCTLIRSSKDVAKPYIEPLLENVLLPKF

                                                                      888
AKROVALWTLGOLVASTGYVVEPYRKYPTLEEVLLNFLKTEONQGTRREAIRVL           870
FKRDAALTLLGOLAASSGYVVGPLLDYPELLGILINILCKTENNPHIRRGTVRLI          861
FKREAALKALGOLAASSGYVIDPLLLDMPELLGILVNILKTIENSQNIRROTVTLI
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FIG. 4C

FIG. 4D

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NLPL-DEFYPAYSMVALMRIFRDQSLSHHTMVVQAITFIFKSLGLKCVQFLPQ      992
VSPSNDEYYLTVVIHNLMKILNDPSLSIHHTAAIQAIMHIFQNLGLRCVSFLDQ     963
MSPSNDEYYTTVVIHCLLKILKDPSLSSSYHTAVIQAIMHIFQTLGLKCVSFLDQ    954

MNTSIOSTILLIEOIVVALGGEFKLYLPOLIPHMERVFMHDNSQGRIVSIKLL      1097
I-IKLQITIISVIESISKALEGEFKRFVPETLTFFLEDILENDQSNKRIVPIRIL    1067
V-AKLOITLVSVIEAISKALEGEFKRLVPLTLTLFLEVILENDKSSDKVLSRRVL    1058

TDYASRIIHPIVRTLDO--SPEERSTAMDTLSSLVFQLGKKYOIFIPMVNKVLV     1200
SEMSSRIVOALVRILNNGDR-ELTKATMNTLSLLLOLGTDFVVFVPVINKALL      1169
FEMSSRIVHSLLRVLSSTTSDELSKVIMNTLSLLLIOMGTSFALFIPVINEVLM     1161

ALASGPVETGPMKKLHVSTINLOKAWGAARRVSKDDWLEWLRRUSLELLKDSSS     1297
-------------VTKLPVNONILKNAWYCSOOKTKEDWOEWIRRLESIOLLKESPS  1257
----------VAKLPINOSVLKSAWNSSOORTKEDWOEWSKRLESIOLLKESPS      1250

QDIAEVTOTLLLNLAEFMEHSDKGPLLPLRDDNGIVLLGGERAAKCRAYAKALHKE   1401
ENPPEIYOMLLLNLLVEFMLHDDK-PLPIP-----IHTLGKYAOKCHAFAKALHYKE  1357
LNPPEIHOTLLLNLEVEFMLHDDK--ALPIP------TOSLGEYAERCHAYAKALHYKE 1350

LHEWLEDALVAYDKKMDTNKDDPELMLGRMRCLEALGEWGOLHOOCCEKWTLVND    1506
LORWLEDALAAYNEKEAAGEDSVEVHMGKLRSLYALGEWEELSKLASEKWGTAKP    1461
LERWLEDALHAYNEREKAGDTSVSVTLGKMRSLHALGEWEOLSOLAARKWKVSKL    1454

SLAOOCIDKARDLLDAELTAMAGESYSRAYGAMVSCHMLSELLEEVLOYKLVP-    1609
KKAEVHIIFNARDLLVTELSALVNESYNRAYNVVVRAOIIAELEEIIKYKKLPON   1566
DNASKHILNARDLLVTEISALINESYNRAYSVIVRTOIITEFEEIIKYKOLRPPN   1559

CGKSGRLALAHKTLVLLE--GVDPSROLDHP-LPTVHPOVTYAYMKNMWKSARK    1710
CRKSGRMALAKKVLNTLLEETDDP-----DHPNTAKASPPVVYAOLKYLWATGLQ  1667
CRKSGRMRLANKALNMLLEGGNDP------SLPNTVKAPPPVVYAOLKYIWATGAY 1660

LMARCFLKLGEWOLNLOGINESTIPK-VLEOYYSAATEHDRSWYKAWHAWAVMNF  1798
LLARCFLKOGEWRVCLOPKWRLSNPDSILGSYLLATHFDNTWYKAWHNWALANF   1767
LLARCFLKOGEWRIATOPNWRNTNPDAILGSYLLATHFDKNWYKAWHNWALANF   1760
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FIG. 4E

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RAFT1   EAVLHYKHQNQARDEKKKLRHASGANITNATTIATIAASAAAATSTEGSNS
TOR2    EVISMLTSVSK--KKQE------------GSDASSVTDIN-EFDNGMIGVNT
TOR1    EVISMVQEETKLNGGKND----------DDDDTAVNNDNVRIDGSILGSGS

RAFT1   LRVLLWFDYGHWPDVNEALVEGVKALQIDTWLQVIPQLARIDTPRPLVG
TOR2    ERLLLWFTFGGIPEATQAMHEGFNLIQIGTWLEVLPQLISRIHQPNQIVS
TOR1    ERLLLELFNFGGIKEVSQAMYEGFNLMKIENWLEVLPQLISRIHQPDPTVS

RAFT1   AMMVSEELIRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMME
TOR2    AELVSHELIRMAVLWHEQWYEGLDDASRQFFGEHNTEKMFAALEPLYEMLK
TOR1    AELVSHELIRVAVEWHELWYEGLEEDASRQFEVEHNIEKMFSTLEPLHKHLG

RAFT1   QLPQLTSLELQYVSPKLLMCRDEELAVPGTYDPN-QPIIRIQSIAPSLQVI
TOR2    QLPQLQTLELQHVSPKLLSAHDLEELAVPGTRASGGKPIVKISKFEPVFSVI
TOR1    QIPQLQTLDLQHVSPQLLATHDLLELAVPGTYFP-GKPTIRIAKFEPLFSVI

RAFT1   KNLSIQRYAVIPLSTNSGLIGWVPHCDTLHAEIRDYREKKKILLNIEHRIM
TOR2    RHLDJQQYPAIPLSPKSGELGWVPNSDTFHVEIREHREAKKIPLNIEHWVM
TOR1    RHLDJQQYPAIPLSPKSGLLGWVPNSDTFHVEIREHRDAKKIPLNIEQWVM

RAFT1   SLAVMSMVGYILGLGDRHPSNLMLDRLSGKILHIDFGDCFEVAMTREKFPE
TOR2    SLAVMSMTGYILGLGDRHPSNLMLDRITGKVIHIDFGDCFEAAILREKFPE
TOR1    SLAVMSMTGYILGLGDRHPSNLMLDRITGKVIHIDFGDCFEAAILREKYPE

RAFT1   NWRLMDTNAKGNKRSRTRTDSYSAGOSVEILDGVELLDGVELGEPAHK---KTGTTV
TOR2    NW--------------------------------GFDL--PTKKIEEETGIQL
TOR1    HW--------------------------------GFDL--PPOKLTEQTGIPL

RAFT1   DTLDVPTQVELLIKQATSHENLCQCYIGWCPFW
TOR2    NDLDVPEQVDKLIQQATSVENLCQHYIGWCPFW
TOR1    NELDVPEQVDKLIQQATSIERLCQHYIGWCPFW
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FIG. 4F

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ESEAESNESSPTPSPLQKKVTEDLSKTLLLYTVPAVQGFFRSISLSRGNNLQDT   1903
---FDAKEVHYSSNLIHRHV--------IPAIKGEFHSISLSLSESSLQDA      1843
---LTINGNRYPLELIQRHV--------VPAIKGEFHSISLLETSCLQDT       1840

RLIHQLLTDIGRYHPQALIYPLTVASKSTTTARHNAANKILKNMCEHSNTLVQQ  2008
RSLLSLLSDLGKAHPQALVYPLMVAIKSESLSRQKAALSIIEKMRIHSPVLVDQ  1948
NSLLSLLSDLGKAHPQALVYPLTVAIKSESVSRQKAALSIIEKIRIHSPVLVNQ  1945

RGPQTLLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISK 2113
RGPETLREISFQNSFGRDLNDAYEWLMNYKKSKDVSNLNQAWDIYYNVFRKIGK  2053
NEPQTLSEVSFQKSFGRDLNDAYEWLNNYKKSKDINNLNQAWDIYYNVRKITR   2050

TSKQRPRKLTLMGSNGHEFVFLLKGHEDLRQDERVMQLFGLVNTLLANDPTSLR  2217
SSKQRPRKFCIKGSDGKDYKYVLKGHEDIRQDSLVMQLFGLVNTLLQNDAECFR  2158
SSKQRPRKFSIKGSDGKDYKYVLKGHEDIRQDSLVMQLFGLVNTLLKNDSECFK  2154

LRMAPDYDHLTLMQKVEVEEHAVNNTAGDDLAKLLWLKSPSSEVWFDRRTNYTR  2322
LQMAPDYDNLTLLQKVEVEFTYALNNTEGQDLYKVLCWLKSRSSETWLERRITYTR 2263
LQMAPDYENLTLLQKIEVEFTYALDNTKGQDLYKILEWLKSRSSETWLERRITYTR 2259

KIPFERLTRMLTNAMEVTGLDRNYRTTCHTVMEVLREHKDSVMAVLEAFVYDPLL 2427
KVPFERLTRMLTYAMEVSGIEGSFRITCENVMKVLRDNKGSLMAILEAFAFDPLI 2368
KVPFERLTRMLTYAMEVSGIEGSFRITCENVMRVLRDNKESLMAILEAFALDPLI 2364

PE-SIHSFIGDGLVKPEAL-------NKKAIOIINRVRDKLTGRDFSHD        2517
PVMNANELLSNGAITEEEVORVENEHKNAIRNARAMLVLKRITDKLTGNDIRRF  2441
PLINPSELLRKGAITVEEAANMEAQQNETRNARAMLVLRRITDKLTGNDIKRF  2437

                                                         2550
                                                         2474
                                                         2470
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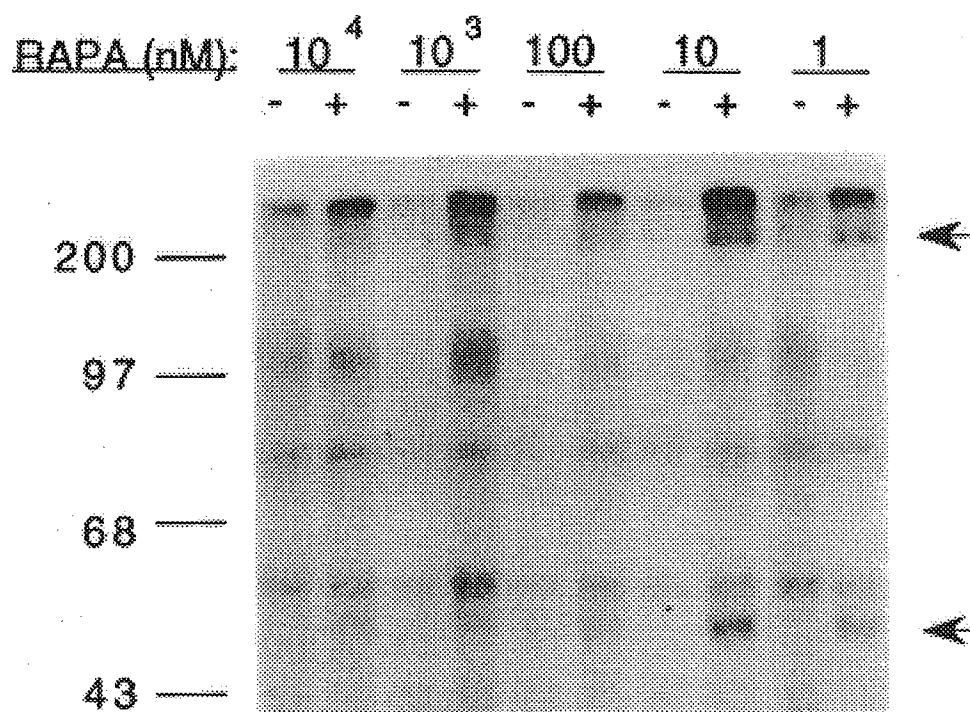
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FIG. 5

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MAMMALIAN PROTEINS THAT BIND TO FKBP12 IN A RAPAMYCIN-DEPENDENT FASHION

BACKGROUND OF THE INVENTION

The natural products cyclosporin A, FK506, and rapamycin are potent immunosuppressants with realized or potential clinical applications in the prevention of graft rejection after organ transplantation and the treatment of autoimmune disorders (Borel, 1986; Kino et al., 1987; Martel et al., 1977). These drugs act by inhibiting intermediate steps in the signaling pathways that effect the T-cell response to antigen (for reviews see, Fruman et al., 1994; Kunz and Hall, 1993; Liu, 1993; Schreiber, 1991). This makes them useful probes for identifying the components of those pathways and determining their physiological roles.

The immunosuppressants interact with the immunophilins, which are small, soluble, receptor proteins that mediate their actions. Cyclosporin A (a cyclical undecapeptide) binds to cyclophilin A, whereas FK506 and rapamycin (two related macrolide antibiotics) bind to a distinct receptor protein, FKBP12 (Handschumacher et al., 1984; Harding et al., 1989; Siekierka et al., 1989). Though cyclophilin and FKBP12 differ markedly in amino acid sequence, both immunophilins have peptidyl-prolyl cis-trans isomerization (rotamase) activity, which is inhibited by their respective ligands (for review, see Heitman et al., 1992). However, this inhibition does not appear to explain the effects of the immunosuppressants (Bierer et al., 1990a, b; Tropschug et al., 1989). Instead, the action of cyclosporin A and FK506 derives from the binding of the drug-receptor complexes to the calcium-activated protein phosphatase, calcineurin (Liu et al., 1991). This association inhibits the catalytic activity of the phosphatase, which is required for the Ca^{++}-dependent initial step in the activation of the T-lymphocyte via the T-cell receptor (Flanagan et al., 1991; Kronke et al., 1984).

On the other hand, rapamycin appears to block a later, Ca^{++}-independent stage in the T-cell response. This drug selectively inhibits the IL-2 stimulated G1 to S cell-cycle transition that initiates T-cell proliferation (Dumont et al., 1990b). Although this inhibition has been correlated with the decreased activity of the 70 kDa S6 kinase ($pp70^{S6K}$), a known downstream effector of the IL-2 receptor, the FKBP12-rapamycin complex does not appear to interact directly with this kinase (Chung et al., 1992; Kuo et al., 1992). Similarly, in T-cells and other cell types, rapamycin blocks progression of the cell cycle by preventing the activation of the cyclin-dependent kinases $p33^{cdk2}$ and $p34^{cdc2}$, but an association of the drug-immunophilin complex with the kinases or their respective cyclins has not been demonstrated (Albers et al., 1993; Jayaraman and Marks, 1993; Morice et al., 1993).

In the budding yeast *S. cerevisiae*, rapamycin also causes an arrest in the G1 phase of the cell cycle through its binding to a highly conserved FKBP12 homologue (Heitman et al., 1991b). The toxicity of the drug for yeast cells has allowed, through genetic selection, the identification of two homologous genes, which, when mutated, render the cells rapamycin-resistant (Heitman et al., 1991a). This led to the proposal that the products of these genes, which show some

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amino acid homology to the catalytic domain of the p110 subunit of PI-3 kinase, are the Targets Of Rapamycin and hence to the designation of the genes as TOR1 and TOR2 (Kunz et al., 1993). Direct support for this proposal, however, has not been presented and how the TOR gene products confer sensitivity to rapamycin remains to be elucidated. Alternatively, it has been suggested that in the signaling pathway blocked by rapamycin, the TOR proteins, like the S6 kinase and the cyclin-dependent kinases, lie downstream from the direct target of the FKBP12-rapamycin complex (Albers et al., 1993; Helliwell et al., 1994). This model assumes that the TOR mutations lead to the constitutive activation of the TOR1 and TOR2 proteins.

Besides binding to calcineurin in a FK506-dependent manner, FKBP12 can also interact with calcium-channel proteins, the ryanodine receptor, which mediates calcium induced calcium released (Jayaraman et al., 1992; Timerman et al., 1993) and the inositol 1,4,5,-triphosphate (IP_3) receptor (A. Cameron, A. Kaplin, D. Sabatini, J. Steiner, S. Snyder, unpublished). These associations do not require FK506 or rapamycin; indeed these drugs dissociate the FKBP12-channel complex.

There is a need in the art to identify, isolate, and purify the mammalian cellular proteins that interact with FKBP12 only in the presence of rapamycin. Such proteins play a role in immunological, neurological, and cell cycle functions.

SUMMARY OF THE INVENTION

It is an object of the invention to provide isolated, purified cDNA molecules encoding rapamycin and FKBP target molecules.

It is another object of the invention to provide fusion proteins comprising rapamycin and FKBP targets.

It is still another object of the invention to provide an isolated and purified rapamycin and FKBP target molecule.

It is still another object of the invention to provide an expression construct which directs synthesis in a cell of an RNA molecule which inibits expression of a rapamycin and FKBP target molecule.

It is yet another object of the invention to provide isolated, purified cDNA molecules which are complementary to genes encoding rapamcyin and FKBP target molecules.

It is an object of the invention to provide a method of screening for potential therapeutic agents.

It is another object of the invention to provide a method of purifying a rapamycin and FKBP target molecule.

It is still another object of the invention to provide a method of isolating DNA sequences which code for rapamycin and FKBP target molecules.

These and other objects of the inveniton are provided by one or more of the embodiments described below. In one embodiment of the invention an isolated, purified cDNA molecule is provided which encodes RAFT1, a protein having the amino acid sequence shown in SEQ ID NO:1.

In another embodiment of the invention a fusion protein comprising the amino acid sequence shown in SEQ ID NO:1, is provided.

In yet another embodiment of the invention an isolated and purified RAFT1 protein having the amino acid sequence shown in SEQ ID NO:1 is provided. Also provided is an isolated and purified RAFT2 protein, having an apparent molecular weight on SDS polyacrylamide gels of 35 kDa. Also provided is an isolated and purified mammalian RAFT protein which is free of proteins which do not bind to rapamycin and FKBP12. Also provided is a mammalian RAFT protein prepared by the process of:

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contacting a preparation of mammalian proteins with FKBP12 in the presence of rapamycin;

isolating mammalian proteins which bind to FKBP12 in the presence of rapamycin from those mammalian proteins which do not bind; and

dissociating bound mammalian proteins from FKBP12 to provide a mammalian RAFT protein.

In still another embodiment of the invention an expression construct is provided. The expression construct comprises a promoter operably linked to at least 20 nucleotides of the antisense strand of RAFT1 cDNA, said expression construct directing synthesis in a cell of an RNA molecule which is complementary to RAFT1 RNA.

In another embodiment of the invention an isolated, purified cDNA molecule comprising at least 20 nucleotides of the sequence of RAFT1 is provided.

In yet another embodiment of the invention a method of screening substances for potential as therapeutic agents is provided. The method comprises the steps of:

contacting a substance to be tested with three components: (a) FKBP12, (b) rapamycin, and (c) a protein selected from the group consisting of RAFT1 and RAFT2;

determining the amount of one of said components bound to the other components in the presence and absence of said substance; a substance which increases or decreases the amount of said component bound being a potential therapeutic agent.

In one embodiment of the invention a method of purifying a mammalian RAFT protein is provided. The method comprises the steps of:

contacting a preparation of mammalian proteins with FKBP12 in the presence of rapamycin;

isolating mammalian proteins which bind to FKBP12 in the presence of rapamycin from those mammalian proteins which do not bind;

dissociating bound mammalian proteins from FKBP12 to provide a mammalian RAFT protein.

In another embodiment of the invention methods of isolating mammalian RAFT DNA sequences are provided. One of the methods comprises:

probing a library of mammalian DNA sequences with a probe which comprises at least 15 contiguous nucleotides selected from the sequence of RAFT1 cDNA.

Another of the methods comprises:

amplifying a DNA sequence using at least one primer which comprises at least 10 contiguous nucleotides selected from the sequence of RAFT1 cDNA.

These and other embodiments of the invention provide the art with potent tools for identifying drugs useful in the treatment of immunological, neurological, and cell cycle-related diseases and defects.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows partial purification of the FKBP12-rapamycin target proteins from brain cytosol by heparin column chromatography.

A cytosolic fraction prepared from a rat brain homogenate was applied to a heparin column. The material that remained bound to the column after washing with 5 column volumes of wash buffer containing 200 mM KCl, was eluted with a linear gradient from 200 mM to 600 mM KCl in homogenization buffer. Aliquots of the crude cytosol (S), the column flow through (FT), and the wash (W) were tested in the crosslinking assay with (+) or without (−) rapamycin

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(100 nM). Every other fraction eluted from the heparin column was tested in the crosslinking assay in the presence of 100 nM rapamycin. No rapamycin specific crosslinked products are visible in the crude cytosol because of the high concentrations of endogenous FKBP12 present in the initial sample.

FIG. 2 shows FK506 and unlabeled FKBP12 prevent the rapamycin-dependent association of ^{32}P-FKBP12 to the target proteins.

FIG. 2A) The heparin column eluate containing the RAFTs was tested in the crosslinking assay at the indicated concentrations of rapamycin with or without the addition of 1 μM FK506 or 1 μM FKBP12. FIG. 2B) Neither FK506 alone nor the ethanol vehicle induce crosslinking of FKBP12 to RAFT. The heparin eluate containing RAFT was tested in the crosslinking assay with the indicated concentrations of FK506 or ethanol. This experiment was repeated twice with identical results.

FIG. 3 shows purification of RAFT1 with a FKBP12-rapamycin affinity column.

RAFT enriched fractions eluting from the heparin column between 300 and 450 mM KCl, were incubated in the presence (+) or absence (−) of 100 nM rapamycin with GST-(PKA)2-FKBP12 fusion protein (20 μg) immobilized on glutathione agarose beads. The material that remained associated with the beads after extensive washes was analyzed by SDS-PAGE (8%) and silver staining. RAFT1 is present only in the sample treated with rapamycin. The large band at 36 kDa is the GST-FKBP12 fusion protein.

FIGS. 4A through 4F shows alignment of RAFT1 amino acid sequence (SEQ ID NO:1) with the predicted amino acid sequences of TOR2 (SEQ ID NO:3) and TOR1 (SEQ ID NO:2).

The alignment was maximized by introducing insertions marked by dashes. Sequences in RAFT1 identical to TOR2 and/or TOR1 are indicated with gray shading. The sequences of tryptic peptides obtained by microsequencing are indicated with a line above the RAFT1 sequence. Sequences used to design primers for PCR are indicated with an arrow above the residues (direction indicate sense or antisense). The PKC site conserved between RAFT1, TOR1 and TOR2 is boxed.

FIG. 5 shows rapamycin-dependent crosslinking of FKBP12 to two PC12 cell cytosolic proteins of approximate molecular weight 245 kDa and 35 kDa.

^{32}P-labeled FKBP12 (10^5 cpm) was incubated with cytosolic fractions from PC12 cells with or without the indicated concentration of rapamycin for 1 hr. at 4° C. The crosslinker DSS was then added and the incubation continued for 40 minutes before processing for SDS-PAGE (4%–12% gradient) and autoradiography. The arrows indicate the two bands that appear only in the presence of rapamycin. This experiment was repeated three times with identical results.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have isolated and identified proteins, which we designate RAFT1 and RAFT2, that interact with the FKBP12-rapamycin complex. Rapamycin-induced binding of FKBP12 to RAFT1 occurs at drug concentrations as low as 1 and 10 nM, resembling pharmacological potency in vivo (Bierer et al., 1990a; Dumont et al., 1990a). FK506 and rapamycin bind with similar affinities to the same binding site on FKBP12 and antagonize each others' actions in vivo (Bierer et al., 1990a; Dumont et al., 1990b). Consistent with these facts, FK506 does not induce interactions between FKBP12 and RAFT1 but, instead, prevents the rapamycin-

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mediated effect. Since rapamycin has pleiotropic effects on a wide variety of cell types, the target of its complex with FKBP12 is likely to be an early participant in several signal transduction pathways.

We have also isolated and purified a cDNA molecule which encodes RAFT1. The predicted amino acid sequence of the protein, which exactly corresponds to the empirically determined amino acid sequences of tryptic peptides of RAFT1, is shown in SEQ ID NO:1. The cDNA sequence can be used to express in recombinant cells RAFT1 proteins or portions of the RAFT1 protein. Similarly, the cDNA sequence can be used to construct fused genes which will express fusion proteins comprising all or part of the RAFT1 sequence. Having provided the art with the amino acid sequence of the RAFT1 protein, other coding sequences can be devised which differ from that isolated virtue of the degeneracy, of the genetic code. Such nucleotide sequences are within the scope of the present invention.

RAFT1 has an apparent molecular weight on SDS polyacrylamide gels of 245 kDa. RAFT2 has an apparent molecular weight on SDS polyacrylamide gels of 35 kDa. Isolated and purified RAFT1 protein can be obtained by means of recombinant DNA technology or by isolating and purifying the protein directly from natural sources. One means of purifying RAFTs involves contacting a preparation of mammalian proteins with FKBP12 in the presence of rapamycin. Those proteins which bind to FKBP12 in the presence of rapamycin can then be separated from those which do not bind. Bound proteins can then be dissociated to yield a preparation of a RAFT protein. It is convenient if the FKBP12 is immobilized, for example, on a solid support. One convenient means is to immobilize FKBP12 on a column-packing matrix. For example, an FKPB12-glutathione-S-transferase fusion protein can be readily bound to glutathione-agarose to provide immobilized FKBP12. Another means of purifying RAFT proteins is by use of a heparin chromatography column. The RAFT proteins bind to the heparin and can be eluted at 300 to 450 mM KCl.

Because of the role of rapamycin in immunological, cell cycle, and neurological functions, it may be desirable to inhibit the expression of RAFT1. One means to accomplish this is to use antisense polynucleotides. Antisense polynucleotides can be made synthetically. Alternatively, expression constructs may be used which comprise a promoter operably linked to at least 20 nucloetides of the antisense strand of RAFT1 cDNA. The expression construct directs the synthesis in a cell of an RNA molecule which is complementary to RAFT1 mRNA. Any suitable promoter can be used, depending on the cell system in which expression of the antisense molecule is desired.

The nucleotide sequence of RAFT1 can be used to generate probes which comprise at least 15–20 nucleotides of the recited sequence. These probes can be used to screen a library of mammalian DNA molecules. Techniques for making nucleotide probes and screening genomic or cDNA libraries are well known in the art. Alternatively, other RAFT nucleotide sequences can be obtained by amplification of mammalian DNA using as primers one or two polynucleotides comprising at least 10 contiguous nucleotides selected from the sequence of RAFT1. Techniques for amplification of DNA are also well known in the art.

RAFT1 and RAFT2 can be used to screen substances for potential as therapeutic agents for immunological, cell cycle, and neurological disease states. As described here, rapamycin, FKBP12, RAFT1, and RAFT2 bind to each

other and form a complex. Test compounds can be screened for potential therapeutic utility by contacting a test compound with three components: (a) FKBP12; (b) rapamycin; and (c) a protein selected from the group consisting of RAFT1 and RAFT2. The amount of one of the components in the complex is determined, in the presence and in the absence of the substance to be tested. A substance which increases or decreasees the amount of the component in the complex is a potential therapeutic agent. Means used for determining amounts of components can be any known in the art, including the use of radioactive components, antibodies specific for components, densitometry, etc.

EXAMPLES

The following materials were used in the examples described below. Frozen rat brains stripped of the meninges were obtained from Harlan Bioproducts (Indianapolis, Ind.). Other materials were purchased from the following sources: $[\gamma\text{-}^{32}P]$-ATP (NEG-02z) from New England Nuclear (Cambridge, Mass.), glutathione-agarose, heart muscle kinase (PKA, #P2645), and heparin-agarose from Sigma Chemical (St. Louis, Mo.), thrombin and antithrombin from Boehringer Mannheim (Indianapolis, Ind.), and disuccinimidyl suberate (DSS) from Pierce (Rockford, Ill.). Rapamycin was a gift of the Wyeth-Ayerst company (Philadelphia, Pa.) and FK506 a gift of the Fujisawa company (Tsukuba City, Japan).

Example 1

Rapamycin Promotes the Binding of FKBP12 to Two Cytosolic Proteins of Mr 245 and 35 kDa

A ^{32}P-radiolabeled FKBP12 probe was used to detect proteins that associate with the immunophilin in the presence of ligand, and are crosslinked to it by the bivalent reagent DSS. The probe was prepared by phosphorylating with $[\gamma~^{32}P]ATP$ a recombinant rat FKBP12 to which two consensus sites for cyclic AMP-dependent protein kinase (PKA) were added at the N-terminus (Blanar and Rutter, 1992; Li et al., 1992). Since this modification did not alter the capacity of the protein to associate with calcineurin in the presence of FK506. the probe can be used to identify a target of the FKBP12-rapamycin complex.

PC12 pheochromocytoma cell cytosolic extracts were incubated with ^{32}P-FKBP12 in the presence or absence of rapamycin and then treated with the crosslinker DSS before gel electrophoretic analysis followed by autoradiography. The drug caused the formation of two protein complexes with radioactive FKBP12, corresponding to bands of Mr 260 and 50 kDa (FIG. 5). Taking into account the 15 kDa Mr of the modified FKBP12 probe, the crosslinked proteins were estimated to be 245 kDa and 35 kDa, respectively. The crosslinked complexes were observed over a wide rapamycin concentration range, but were more prominent at the low concentrations of 1 and 10 nM, possibly because of an inhibitory effect on the association of the higher amounts of ethanol (the solvent of the drug) present at the higher drug concentrations (FIG. 5). Rapamycin also induced the formation of similar complexes when the probe was incubated with cytosolic extracts from several rat tissues, including liver, kidney, heart, small intestine, thymus, testes, spleen and brain, but no significant differences in abundance of the crosslinked proteins between the tissues were observed. For convenience, further experiments were carried out with whole brain extracts.

The formation of the rapamycin-dependent complexes was specific for FKBP12, since in similar experiments with

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the related immunophilin ^{32}P-FKBP25, no ligand induced complexes were observed.

PC12 cells were maintained in culture as described (Altin et al., 1991). PC12 cells were lysed in homogenization buffer with 0.3% NP-40 instead of CHAPS. Lysis was accomplished in 2 ml buffer/T-150 flask by repeated vortexing at 4° C. Cell debris was sedimented by centrifugation for 10,000×g for 10 minutes at 4° C.

The labeled, cleaved FKBP12 was diluted to 10,000 cpm/ml in 50 mM Hepes pH 7.5, 1 mg/ml BSA. 10 μl of labeled protein (100,000 cpm total), 10 μl of tissue or PC12 cell extract, and 10 μl of drug dilutant buffer (20 mM Hepes 6.8, 100 mM KCl, 1 mM EGTA, 1 mM DTT) containing either 3-fold the desired final concentration of rapamycin, FK506, or equivalent amounts of ethanol, were mixed and incubated for 1 hour at 4° C. After this incubation, 1 ml of 5.5 mg/ml disuccinimidyl suberate (DSS) was added and the incubation continued for 40 minutes. The reaction was terminated by adding one column volume of 2×concentrated sample buffer (Laemmli, 1970) containing 50 mM Tris pH 7.4 and processed by SDS-PAGE (10%, unless otherwise specified) and autoradiography.

Example 2

Specificity of the Rapamycin Induced Association: the Interaction of ^{32}P-FKBP12-rapamycin with the 245 and 35 kDa Proteins is Competed by FK506 and by Unlabeled FKBP12

To investigate further the specificity of the interaction of ^{32}P-FKBP12-rapamycin with the cytosolic proteins, we performed a partial purification to remove endogenous FKBP12, which is present in brain at high concentrations (Steiner et al., 1992). This was accomplished by chromatography on a heparin column, to which the cytosolic proteins that interact with FKBP12-rapamycin bound and could be eluted at 300 to 450 mM KCl (FIG. 1). Free FKBP12, on the other hand, was recovered in the flow-through of this column, as demonstrated by binding to [³H]FK506 (data not shown).

The rat brain extract was applied to a heparin column (2 ml of packed heparin-agarose per brain) at a flow, rate of 1.5 ml/min. The column was washed with 10 column volumes of buffer (20 mM Hepes pH 6.8, 200 mM KCl, 1 mM EGTA, 50 mM NaF, 1.5 mM Na₃VO₄, 4 mM DTT, 1 mM PMSF) and the same protease inhibitors as in the homogenization buffer. The material bound to the column was eluted with a linear KCl gradient from 200 to 600 mM in homogenization buffer. Aliquots (10 μl) of the fractions collected were tested in the crosslinking assay and positive fractions were pooled and concentrated in a centriprep-100 (Amicon, Beverly, Mass.) to ⅓ starting volume. The flowthrough of the heparin column was assayed for the presence of FKBP with a ³H-FK506 binding assay, as described (Steiner et al, 1992).

FK506 antagonizes actions of rapamycin, and both drugs compete for the same binding site on FKBP12 (Bierer et al., 1990a; Dumont et al., 1990a). Accordingly, we examined the influence of FK506 on the rapamycin-induced interaction of ^{32}P-FKBP12 with its putative cytosolic targets. At concentrations ranging from 1 nM to 1 μM rapamycin induced the appearance of intense bands representing crosslinked proteins and, at all rapamycin concentrations tested, this effect was antagonized by 1 μM FK506 (FIG. 2A). As expected for ligands of similar affinity for FKBP12, when equal concentrations (1 μM) of rapamycin and FK506 were present, the intensities of the crosslinked bands were reduced by

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approximately 50% and the reduction progressively increased with increasing ratios of FK506/rapamycin. The heparin column eluate apparently contains limiting amounts of the putative targets of the FKBP12-rapamycin complex, since excess unlabeled FKBP12 (1 μM) completely suppressed the appearance of the crosslinked bands containing labeled FKBP12 (FIG. 2A).

Control experiments (FIG. 2B) confirmed the specificity of the rapamycin effect since the formation of the complex was not induced by several concentrations of FK506 or by ethanol, the solvent of the drugs. These experiments demonstrate that the crosslinked proteins are specific targets of the FKBP12-rapamycin complex and not of the FKBP12-FK506 complex, nor of FKBP12 alone. Therefore, we designate the crosslinked proteins RAFT1 (245 kDa) and RAFT2 (35 kDa) for Rapamycin And FKBP12 Target.

We attempted to separate RAFT1 and RAFT2 under nondenaturing conditions by several chromatography and gel filtration procedures. including DEAE and CM cellulose, reactive dye green 5, and Superose 6 (data not shown). All of these efforts failed, suggesting that RAFT1 and RAFT2 are part of a complex, although it is possible that RAFT2 is a proteolytic fragment of RAFT1 that contains the FKBP12-rapamycin binding site and remains tightly bound to the rest of the polypeptide.

Example 3

Purification of RAFT1

We purified RAFT1 from the heparin column eluate based on its affinity for FKBP12-rapamycin. We constructed a glutathione-S-transferase-FKBP12 fusion protein by cloning, in frame downstream of GST, a cDNA encoding FKBP12 with two N-terminal PKA consensus sites (Smith and Johnson, 1988; Blanar and Rutter, 1992; Li et al., 1992). The encoded protein was expressed in bacteria, purified and immobilized on glutathione-agarose beads. SDS-PAGE analysis of the beads recovered after incubating them with the heparin eluate in the presence or absence of rapamycin shows that the drug induces the binding to the beads of a protein of 245 kDa (FIG. 3). With this simple purification scheme we were able to purify about 5 μg of RAFT1. A low transfer efficiency to nitrocellulose membrane resulted in only 2.5 μg being available for protein sequencing, which corresponds to 10 picomoles of a protein of this size.

Standard techniques of molecular biology cloning were used as described (Sambrook et al., 1989) for the preparation of GST-(PKA)₂-FKBP12 and GST-(PKA)₂-FKBP25 fusion proteins, unless otherwise specified. All cDNAs obtained with the polymerase chain reaction were sequenced using the Sequenase kit (Amersham, Arlington Heights, Ill.). cDNAs for the rat FKBP12 and FKBP25 were obtained with the PCR using 5' and 3' primers to the corresponding human FKBP12 (Standaert et al., 1990) or FKBP25 (Jin et al., 1992) sequences. The cDNAs were cloned into pBluescript (Stratagene, La Jolla, Calif.).

A 5' primer (PKA-12-1 or PKA-25-1) encoding a BamHI site, two consensus PKA phosphorylation sites (Blanar and Rutter, 1992; Li et al., 1992), and the first 6 amino acids of FKBP12 or FKBP25 was used with a 3' primer (PKA-12-2 or PKA-25-2) encoding an EcoRI site and the last 6 codons of FKBP12 or FKBP25 in a PCR with Vent Polymerase (New England Biolabs, Beverly, Mass.) using the rat FKBPs cDNAs cloned in pBluescript as templates. The amplified DNA fragments were gel purified, digested with BamnH1 and EcoR1 and cloned into the pGEX-2T vector (Pharmacia,

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Upsala, Sweden) that had been linearized with the same restriction enzymes. The resulting construct was used to transform BL21 (DE3) *E. coli* (Novagen, Madison, Wis.) in which expression can be induced with IPTG. The primer sequences were is follows:

PKA-12-1:5' CCGGATCCCGTCGAGCTTCAGT-TGAACTACGGCGTGC TTCTGTAGCCATGG-GAGTGCAGGTGGA 3' (SEQ ID NO:4)

PKA-12-2: 5' GGCCGGAATTCTCATTCCAGTTTTA-GAA 3' (SEQ ID NO:5)

PKA-25-1: 5' CCGGATCCCGTCGAGCTTCAGT-TGAACTACGGCGTGC TTCTGTAGCCATGGCG-GCGGCCGTTCC 3' (SEQ ID NO:10)

PKA-25-2: 5' GGCCGGAATTCTCAATCAATATC-CACTA 3' (SEQ ID NO:11)

The fusion proteins were purified with glutathione-agarose as previously described (Smith and Johnson, 1988) from bacterial cultures induced with 1 mM IPTG.

The concentrated heparin column eluate was incubated for 2 hours at 4° C. with 1/50 volume of glutathione-agarose to remove endogenous glutathione binding proteins. The beads were removed by centrifugation at 1000×g for 3 minutes. Fresh glutathione-agarose (1/500 volume) and 20 μg of purified GST-PKA-FKBP12 fusion protein were then added to the cleared heparin column eluate with or without 100 nM rapamycin. After a 1 hour incubation at 4° C., the bead were washed 5× with 1.5 ml ice-cold PBS containing 1% Triton X-100 and 500 mM NaCl. The beads were transferred to 3×volume SDS-PAGE sample buffer, and the eluted proteins fractionated by SDS-PAGE and the gel silver stained.

Whether RAFT2 was also bound to the beads could not be determined in this experiment, because its presence would be masked by the large band of similar Mr corresponding to the GST-(PKA)$_2$-FKBP12 fusion protein. When smaller fusion proteins, such as an epitope-tagged FKBP12, were employed for the affinity matrix, the binding of the 35 kDa RAFT2 could also be observed.

The immunophilin fusion proteins containing N-terminal phosphorylation sites for PKA were labeled with a modification of published procedures (Blanar et. al., 1992, Li et. al., 1992). 10 ng of purified GST-PKA-FKBP12 or 25 was mixed with 40 units of PKA and 100 mCi of [γ-P^{32}]-ATP in a buffer containing 20 mM Hepes pH 7.7, 100 mM NaCl, 12 mM MgCl$_2$, 1 mM DTT.

After a 1.5 hour at 37° C. the incubation mixture containing labeled fusion protein was dialyzed twice against 1 L of thrombin cleavage buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2.5 mM CaCl$_2$). The labeled fusion protein was cleaved by adding an equal volume of thrombin cleavage buffer containing 2 mg/ml thrombin and incubating at room temperature for 2 hours. The thrombin was inactivated by adding an equal volume of a stop solution consisting of 1 mM DTT, 1 mM PMSF, 100 units/ml antithrombin III. The specific activity of the probes was estimated at 1×10^5 cpm/pmol of the protein.

Example 4

Protein Sequencing of RAFT1: Homology to TOR1 and TOR2

Affinity purified RAFT1 was separated by SDS-polyacrylamide gel electrophoresis from other proteins that adsorbed to the glutathione-agarose beads, transferred to nitrocellulose membrane, and digested with trypsin. Fractionation of the tryptic digest by narrow-bore reverse phase

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chromatography yielded a complex pattern of over a hundred peaks whose purity was assessed by mass spectroscopy. In most cases, the peaks exhibited multiple mass to charge peak values and it was necessary to rechromatograph these peak fractions on a microbore columns of different selectivity.

For protein sequence analysis affinity purified material derived from 50 brains was fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The proteins transferred were visualized by Ponceau S staining, the 245 kDa RAFT1 band excised and processed for internal amino acid sequence analysis, essentially as described (Tempst et al., 1990).

Membrane-bound protein, about 2.5 μg, was subjected to in-situ proteolytic cleavage using 1 μg trypsin (Sequencing Grade; Boehringer-Mannheim) in 25 ml 100 mM NH$_4$HCO$_3$ (supplemented with 10% acetonitrile and 3% Tween-80) at 37° C. for 3 hours. The resulting peptide mixture was reduced and S-alkylated with, respectively, 0.1% β-mercapto ethanol and 0.3% 4-vinyl pyridine, and fractionated by two-dimensional reversed phase HPLC.

For the primary separations, a 2.1 mm Vydac C4 (214TP54) column was used with gradient elution at a flow rate of 100 μl/min. HPLC solvents and system configuration were as described (Tempst et al., 1990), with improved dead volume reduction through the use of glass capillary tubing (Elicone and Tempst, unpublished). Identification of Trp-containing peptides was done by manual ratio analysis of absorbances at 297 and 277 nm, monitored in real time using an Applied Biosystems model 1000S diode-array detector (Tempst et al., 1990). Fractions were collected by hand, kept on ice for the duration of the run and then stored at −70° C. before repurification and/or analysis. An enzyme blank was done on an equally sized strip of nitrocellulose cut from a blank area of the same blot. Repurifications (second dimension LC) were carried out on a 1.0 mm SGE ODS-2 C18 column using the same solvent system but at a flow rate of 30 μl/min. (C. Elicone, M. Lui, S. Geromanos, H. Erdjument-Bromage, P. Tempst, in press). Samples were always acidified (20% TFA final concentration) and then diluted twofold with 0.1% TFA before rechromatography.

Sequences of 23 peptides separated in this fashion were determined by a combination of automatic Edman degradation, matrix-assisted laser desorption mass-spectroscopy, and UV spectroscopy.

Peak fractions over background were analyzed by a combination of automated Edman degradation and matrix-assisted laser-desorption (MALDI-TOF) mass spectrometry (Geromanos et al., 1994; Elicone et al., 1994). After storage, column fractions were supplemented with neat TFA (to give a final concentration of 10%) before loading onto the sequencer disc and mass spectrometer probe tips. Peptide mass analysis (on 2% aliquots) was carried out using a model LaserTec ResearcH MALDI-TOF instrument (Vestec), with a 337 nm output nitrogen laser and 1.2 m flight tube. The matrix was a-cyano-4-hydroxy cinnamic acid, and a 28 kV ion acceleration and 4.3 kV multiplier voltage were used. Laser power and number of acquisitions were adjusted as judged from optimal deflections of specific maxima, using a Tektronix TDS 520 digitizing oscilloscope. M/z (mass to charge) spectra were generated from the time-of-flight files using GRAMS data analysis software. Every sample was analyzed twice, in the presence and absence of a calibrant (25 femtomoles APID), as described (Geromanos et al., 1994). Chemical sequencing (on 95% of the sample) was clone using a model 477A instrument from

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Applied Biosystems (AB). Stepwise liberated PTH-amino acids were identified using an "on-line" 120A HPLC system (AB) equipped with a PTH C18 (2.1×220 mm; 5 micron particle size) column (AB). Instruments and procedures were optimized for femtomole level phenyl thiohydantoin amino acid analysis as described (Tempst and Riviere, 1990; Erdjument-Bromage et al., 1993).

Peptide average isotopic masses were summed from the identified residues (including the presumed ones) using ProComp version 1.2 software (obtained from Dr. P. C. Andrews, University of Michigan, Ann Arbor, Mich.). Peptide sequences were compared to entries in various sequence databases using the National Center for Biotechnology Information (NCBI) BLAST program (Altschul et al. 1990). Lower stringency alignments between all peptides and selected proteins were done using the Lipman-Pearson algorithm, available in the 'Lasergene' software package (DNASTAR).

Several protein sequence databases (PIR, SwissProt, translated Genbank) were searched for sequences that match any of the 23 peptide sequences obtained from microsequencing of RAFT1. While sequence similarities with hundreds of different proteins were obtained for many of the 23 peptides, none perfectly matched with any of the entries in the databases, nor did any protein match more than one or two peptides, other than the yeast proteins TOR1 and TOR2 (Kunz et al., 1993). Strikingly, sixteen out of the 23 peptides of RAFT1 could be aligned with the yeast TOR sequences, with varying degrees of similarity (FIG. 4).

Example 5

Molecular Cloning of RAFT1

To generate a probe for isolating a RAFT1 cDNA two degenerate oligonucleotides were used in a mixed oligonucleotide polymerase chain reaction (PCR) (Gould et al, 1989) with rat brain cDNA as template. The sense primer was made to a peptide sequence (TYDPNQP, SEQ ID NO:6) obtained from microsequencing of RAFT1, while the antisense primer corresponds to a sequence (HIDFGD, SEQ ID NO:7) conserved between TOR1, TOR2, and p110 PI-3 Kinase. From the alignments of the RAFT1 peptides to the TORs, this sequence was expected to be 220 amino acids downstream of that encoded by the sense primer. The predicted 660 bp PCR product was obtained, cloned, and its authenticity was verified by DNA sequencing, which showed that it encoded two other sequenced tryptic peptides. The PCR product was, therefore, used as a probe (3' probe) to screen a rat striatum cDNA library, which yielded a 5.5 kb partial cDNA clone. An antisense oligonucleotide to the extreme 5' end of this cDNA was then used in a PCR reaction with a degenerate sense oligonucleotide to another peptide sequence (NDQVFE, SEQ ID NO:8) obtained from microsequencing. The predicted 1.1 kb PCR product was obtained, cloned and used as probe (5' probe) to screen a rat brainstem cDNA library in parallel with the original 3' probe. Phage plaques that hybridized with both probes were isolated and one was found to carry a 8.6 kb insert. A degenerate sense oligpnucleotide corresponding to the amino acid sequence TYDPNQP (SEQ ID NO:6), which was obtained from microsequencing of RAFT1 and aligns to residues 2086 to 2093 of TOR2, and a degenerate antisense primer corresponding to amino acids 2296 to 2301 (HIDFGD, SEQ ID NO:7) of TOR2 were used in a PCR reaction with rat whole brain cDNA as template. The protocol for the PCR was: an initial 5 min at 94° C., followed by 35 cycles of 94° C. for 40s, 56° C. for 1 min, 72° C. for

1 min, and a final incubation at 72° C. for 5 min. The PCR products were fractionated on a 1.1% agarose gel, the expected 700 bp DNA fragment purified and subcloned into pBluescript.

The RAFT-1 cDNA fragment in pBluescript was amplified by PCR, the product gel purified and labeled by nick translation with a commercial kit (Boehringer Mannheim). This probe (designated 3' probe) was used to screen 1×10⁶ phage plaques of a rat striatum λ ZAP library (Stratagene), as described (Sambrook et al.). Forty seven positive clones were identified and 10 of them were purified by an additional two rounds of screening. None of the inserts contained a complete open reading frame. The 5' end of the largest insert (5.5 kb) was used to design a 18 bp antisense oligdnucleotide (3.1 as) that was used in another PCR reaction with rat whole brain cDNA as template and a degenerate oligonucleotide corresponding to the amino acid sequence NDQVFE (SEQ ID NO:8, part of a peptide obtained from microsequencing) as the sense primer. The PCR products were fractionated on a 1% agarose gel and a DNA fragment of 1.1 kb isolated and cloned into the vector pCR-II using the TA cloning kit (Invitrogen, San Diego, Calif.). The cDNA fragment was amplified by PCR, the product gel purified and labeled by nick translation. This probe (designated 5' probe) was used to screen 1×10⁶ phage plaques from a rat brainstem λ ZAP library. Duplicate filters were screened with the 3' probe. Eight clones hybridized with both the 5' and 3' probes, and five of these were purified through 2 additional rounds of screening. One clone contained a 8.6 kb insert that encodes all 23 peptide sequences obtained by microsequencing.

PCR primer sequences were as follows:

TYDPNQP (SEQ ID NO:6): 5 '-GGGGGATCCACNTA (C/T)GA(C/T)CCNAA(C/T) CA(A/G)C-3' (SEQ ID NO:12)

HIDFGD (SEQ ID NO:7): 5 '-GCGGAATTC(G/A) TCNCC(G/A)AA(G/A)TC(T/G/A) AT(G/A)TG-3' (SEQ ID NO:13)

NDQVFE (SEQ ID NO:8): 5'-GGGGGATCCAA(C/T) GA(C/T)CA(G/A)GTNTT (T/C)GA-3' (SEQ ID NO:14)

3.1as: 5' -GAGCCACCACGATTTGCT-3'(SEQ ID NO:9)

cDNA clones were sequenced using the flourescent terminator method of cycle sequencing on a Applied Biosystems 373a automated DNA sequencer at the DNA analysis Facility of the Johns Hopkins University (Smith et al., 1986; McCombie et al, 1992), or with the dideoxy chain termination method using the Sequenase kit (Amersham, Arlington Heights, Ill.). Oligonucleotides used for sequencing were synthesized on an ABI 394 synthesizer following ABI protocols. DNA sequence data was analyzed using Sequencher software from Gene Codes (Ann Arbor, Mich.). Protein alignments were done with help from the e-mail service of the Computational Biochemistry Research Group (CBRG) at the ETH.

This cDNA contains an open reading frame of 7.6 kb with an initiation methionine codon that conforms to the Kozak consensus sequence (Kozak, 1986) and is preceded by an in-frame termination codon. The protein encoded by this open reading frame contains all 23 peptide sequences obtained by microsequencing of RAFT1 (FIG. 4). Interestingly, none of the peptides sequenced correspond to the C-terminal 250 amino acids of RAFT1, which may indicate that this portion of the protein was proteolytically removed during the purification.

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The RAFT1 cDNA predicts a protein of 2550 amino acids with a molecular mass of 289 kDa and a PI of 6.8. Over its entire sequence RAFT1 is 43% identical to TOR2 and 39% identical to TOR1 (FIG. 4). The C-terminal 600 amino acids of RAFT1, which, by analogy to the TORs (Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994), is predicted to contain lipid kinase activities, is 65% identical to the yeast proteins. The RAFT1 protein has over 20 consensus sites for phosphorylation by protein kinase C (PKC), including one at serine$_{2035}$, which is in the analogous position to the serine (S_{1972} in TOR1 and S_{1975} in TOR2) found mutated to arginine in rapamycin resistant yeast (boxed residues in FIG. 4).

The predicted RAFT1 protein is 80 amino acids longer than the TOR proteins, and contains several regions with no apparent homology to the yeast proteins, the largest being a 93 amino acid insertion corresponding to residues 270 to 363 of RAFT1. It is possible that these regions are generated by alternative splicing of exons that may be tissue specific to the brain. They are unlikely to be the translation product of unspliced introns because they were found in several cDNA clones isolated from different libraries and the DNA sequence does not reveal consensus splice junction sites.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

  (iii) NUMBER OF SEQUENCES: 14

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 2549 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

  (iii) HYPOTHETICAL: YES

   (iv) ANTI-SENSE: NO

   (vi) ORIGINAL SOURCE:
       (A) ORGANISM: Rattus rattus
       (F) TISSUE TYPE: pheochromocytoma
       (G) CELL TYPE: PC12

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Leu Gly Thr Gly Pro Ala Thr Ala Thr Ala Gly Ala Ala Thr Ser
1               5                   10                  15

Ser Asn Val Ser Val Leu Gln Gln Phe Ala Ser Gly Leu Lys Ser Arg
            20                  25                  30

Asn Glu Glu Thr Arg Ala Lys Ala Ala Lys Glu Leu Gln His Tyr Val
        35                  40                  45

Thr Met Glu Leu Arg Glu Met Ser Gln Glu Glu Ser Thr Arg Phe Tyr
    50                  55                  60

Asp Gln Leu Asn His His Ile Phe Glu Leu Val Ser Ser Ser Asp Ala
65                  70                  75                  80

Asn Glu Arg Lys Gly Gly Ile Leu Ala Ile Ala Ser Leu Ile Gly Val
            85                  90                  95

Glu Gly Gly Asn Ser Thr Arg Ile Gly Arg Phe Ala Asn Tyr Leu Arg
            100                 105                 110

Asn Leu Leu Pro Ser Ser Asp Pro Val Val Met Glu Met Ala Ser Lys
        115                 120                 125

Ala Ile Gly Arg Leu Ala Met Ala Gly Asp Thr Phe Thr Ala Glu Tyr
    130                 135                 140

Val Glu Phe Glu Val Lys Arg Ala Leu Glu Trp Leu Gly Ala Asp Arg
145                 150                 155                 160

Asn Glu Gly Arg Arg His Ala Ala Val Leu Val Leu Arg Glu Leu Ala
            165                 170                 175

Ile Ser Val Pro Thr Phe Phe Phe Gln Gln Val Gln Pro Phe Phe Asp
            180                 185                 190

Asn Ile Phe Val Ala Val Trp Asp Pro Lys Gln Ala Ile Arg Glu Gly
        195                 200                 205

Ala Val Ala Ala Leu Arg Ala Cys Leu Ile Leu Thr Thr Gln Arg Glu
    210                 215                 220

Pro Lys Glu Met Gln Lys Pro Gln Trp Tyr Arg His Thr Phe Glu Glu
225                 230                 235                 240

Ala Glu Lys Gly Phe Asp Glu Thr Leu Ala Lys Glu Lys Gly Met Asn
            245                 250                 255

Arg Asp Asp Arg Ile His Gly Ala Leu Leu Ile Leu Asn Glu Leu Val
            260                 265                 270
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Arg Ile Ser Ser Met Glu Gly Glu Arg Leu Arg Glu Glu Met Glu Glu
        275              280              285

Ile Thr Gln Gln Gln Leu Val His Asp Lys Tyr Cys Lys Asp Leu Met
        290              295              300

Gly Phe Gly Thr Lys Pro Arg His Ile Thr Pro Phe Thr Ser Phe Gln
305              310              315              320

Ala Val Gln Pro Gln Gln Ser Asn Ala Leu Val Gly Leu Leu Gly Tyr
                 325              330              335

Ser Ser His Gln Gly Leu Met Gly Phe Gly Ala Ser Pro Ser Pro Thr
                 340              345              350

Lys Ser Thr Leu Val Glu Ser Arg Cys Cys Arg Asp Leu Met Glu Glu
                 355              360              365

Lys Phe Asp Gln Val Cys Gln Trp Val Leu Lys Cys Arg Ser Ser Lys
        370              375              380

Asn Ser Leu Ile Gln Met Thr Ile Leu Asn Leu Leu Pro Arg Leu Val
385              390              395              400

Ala Phe Arg Pro Ser Ala Phe Thr Asp Thr Gln Tyr Leu Gln Asp Thr
                 405              410              415

Met Asn His Val Leu Ser Cys Val Lys Lys Glu Lys Glu Arg Thr Ala
                 420              425              430

Ala Phe Gln Ala Leu Gly Leu Leu Ser Val Ala Val Arg Ser Glu Phe
        435              440              445

Lys Val Tyr Leu Pro Arg Val Leu Asp Ile Ile Arg Ala Ala Leu Pro
        450              455              460

Pro Lys Asp Phe Ala His Lys Arg Gln Lys Thr Val Gln Val Asp Ala
465              470              475              480

Thr Val Phe Thr Cys Ile Ser Met Leu Ala Arg Ala Met Gly Pro Gly
                 485              490              495

Ile Gln Gln Asp Ile Lys Glu Leu Leu Glu Pro Met Leu Ala Val Gly
                 500              505              510

Leu Ser Pro Ala Leu Thr Ala Val Leu Tyr Asp Leu Ser Arg Gln Ile
        515              520              525

Pro Gln Leu Lys Lys Asp Ile Gln Asp Gly Leu Leu Lys Met Leu Ser
        530              535              540

Leu Val Leu Met His Lys Pro Leu Arg His Pro Gly Met Pro Lys Gly
545              550              555              560

Leu Ala His Gln Leu Ala Ser Pro Gly Leu Thr Thr Leu Pro Glu Ala
                 565              570              575

Ser Asp Val Ala Ser Ile Thr Leu Ala Leu Arg Thr Leu Gly Ser Phe
                 580              585              590

Glu Phe Glu Gly His Ser Leu Thr Gln Phe Val Arg His Cys Ala Asp
        595              600              605

His Phe Leu Asn Ser Glu His Lys Glu Ile Arg Met Glu Ala Ala Arg
        610              615              620

Thr Cys Ser Arg Leu Leu Thr Pro Ser Ile His Leu Ile Ser Gly His
625              630              635              640

Ala His Val Val Ser Gln Thr Ala Val Gln Val Ala Asp Val Leu
                 645              650              655

Ser Lys Leu Leu Val Val Gly Ile Thr Asp Pro Asp Pro Asp Ile Arg
                 660              665              670

Tyr Cys Val Leu Ala Ser Leu Asp Glu Arg Phe Asp Ala His Leu Ala
        675              680              685

Gln Ala Glu Asn Leu Gln Ala Leu Phe Val Ala Leu Asn Asp Gln Val
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      690               695               700
Phe Glu Ile Arg Glu Leu Ala Ile Cys Thr Val Gly Arg Leu Ser Ser
705               710               715               720

Met Asn Pro Ala Phe Val Met Pro Phe Leu Arg Lys Met Leu Ile Gln
                  725               730               735

Ile Leu Thr Glu Leu Glu His Ser Gly Ile Gly Arg Ile Lys Glu Gln
                  740               745               750

Ser Ala Arg Met Leu Gly His Leu Val Ser Asn Ala Pro Arg Leu Ile
          755               760               765

Arg Pro Tyr Met Glu Pro Ile Leu Lys Ala Leu Ile Leu Lys Leu Lys
      770               775               780

Asp Pro Asp Pro Asp Pro Asn Pro Gly Val Ile Asn Asn Val Leu Ala
785               790               795               800

Thr Ile Gly Glu Leu Ala Gln Val Ser Gly Leu Glu Met Arg Lys Trp
                  805               810               815

Val Asp Glu Leu Phe Val Ile Ile Met Asp Met Leu Gln Asp Ser Ser
                  820               825               830

Leu Leu Ala Lys Arg Gln Val Ala Leu Trp Thr Leu Gly Gln Leu Val
          835               840               845

Ala Ser Thr Gly Tyr Val Val Glu Pro Tyr Arg Lys Tyr Pro Thr Leu
          850               855               860

Leu Glu Val Leu Leu Asn Phe Leu Lys Thr Glu Gln Asn Gln Gly Thr
865               870               875               880

Arg Arg Glu Ala Ile Arg Val Leu Gly Leu Leu Gly Ala Leu Asp Pro
                  885               890               895

Tyr Lys His Lys Val Asn Ile Gly Met Ile Asp Gln Ser Arg Asp Ala
          900               905               910

Ser Ala Val Ser Leu Ser Glu Ser Lys Ser Ser Gln Asp Ser Ser Asp
          915               920               925

Tyr Ser Thr Ser Glu Met Leu Val Asn Met Gly Asn Leu Pro Leu Asp
      930               935               940

Glu Phe Tyr Pro Ala Val Ser Met Val Ala Leu Met Arg Ile Phe Arg
945               950               955               960

Asp Gln Ser Leu Ser His His His Thr Met Val Val Gln Ala Ile Thr
                  965               970               975

Phe Ile Phe Lys Ser Leu Gly Leu Lys Cys Val Gln Phe Leu Pro Gln
                  980               985               990

Val Met Pro Thr Phe Leu Asn Val Ile Arg Val Cys Asp Gly Ala Ile
          995               1000              1005

Arg Glu Phe Leu Phe Gln Gln Leu Gly Met Leu Val Ser Phe Val Lys
      1010              1015              1020

Ser His Ile Arg Pro Tyr Met Asp Glu Ile Val Thr Leu Met Arg Glu
1025              1030              1035              1040

Phe Trp Val Met Asn Thr Ser Ile Gln Ser Thr Ile Ile Leu Leu Ile
                  1045              1050              1055

Glu Gln Ile Val Val Ala Leu Gly Gly Glu Phe Lys Leu Tyr Leu Pro
          1060              1065              1070

Gln Leu Ile Pro His Met Leu Arg Val Phe Met His Asp Asn Ser Gln
          1075              1080              1085

Gly Arg Ile Val Ser Ile Lys Leu Leu Ala Ala Ile Gln Leu Phe Gly
      1090              1095              1100

Ala Asn Leu Asp Asp Tyr Leu His Leu Leu Leu Pro Pro Ile Val Lys
1105              1110              1115              1120
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Leu Phe Asp Ala Pro Glu Val Pro Leu Pro Ser Arg Lys Ala Ala Leu
             1125                1130                1135

Glu Thr Val Asp Arg Leu Thr Glu Ser Leu Asp Phe Thr Asp Tyr Ala
             1140                1145                1150

Ser Arg Ile Ile His Pro Ile Val Arg Thr Leu Asp Gln Ser Pro Glu
             1155                1160                1165

Leu Arg Ser Thr Ala Met Asp Thr Leu Ser Ser Leu Val Phe Gln Leu
        1170                1175                1180

Gly Lys Lys Tyr Gln Ile Phe Ile Pro Met Val Asn Lys Val Leu Val
   1185                1190                1195                1200

Arg His Arg Ile Asn His Gln Arg Tyr Asp Val Leu Ile Cys Arg Ile
             1205                1210                1215

Val Lys Gly Tyr Thr Leu Ala Asp Glu Glu Glu Asp Pro Leu Ile Tyr
             1220                1225                1230

Gln His Arg Met Leu Arg Ser Ser Gln Gly Asp Ala Leu Ala Ser Gly
        1235                1240                1245

Pro Val Glu Thr Gly Pro Met Lys Lys Leu His Val Ser Thr Ile Asn
   1250                1255                1260

Leu Gln Lys Ala Trp Gly Ala Ala Arg Arg Val Ser Lys Asp Asp Trp
1265                1270                1275                1280

Leu Glu Trp Leu Arg Arg Leu Ser Leu Glu Leu Leu Lys Asp Ser Ser
             1285                1290                1295

Ser Pro Ser Leu Arg Ser Cys Trp Ala Leu Ala Gln Ala Tyr Asn Pro
        1300                1305                1310

Met Ala Arg Asp Leu Phe Asn Ala Ala Phe Val Ser Cys Trp Ser Glu
        1315                1320                1325

Leu Asn Glu Asp Gln Gln Asp Glu Leu Ile Arg Ser Ile Glu Leu Ala
   1330                1335                1340

Leu Thr Ser Gln Asp Ile Ala Glu Val Thr Gln Thr Leu Leu Asn Leu
1345                1350                1355                1360

Ala Glu Phe Met Glu His Ser Asp Lys Gly Pro Leu Pro Leu Arg Asp
             1365                1370                1375

Asp Asn Gly Ile Val Leu Leu Gly Glu Arg Ala Ala Lys Cys Arg Ala
        1380                1385                1390

Tyr Ala Lys Ala Leu His Tyr Lys Glu Leu Glu Phe Gln Lys Gly Pro
   1395                1400                1405

Thr Pro Ala Ile Leu Glu Ser Leu Ile Ser Ile Asn Asn Lys Leu Gln
   1410                1415                1420

Gln Pro Glu Ala Ala Ser Gly Val Leu Glu Tyr Ala Met Lys His Phe
1425                1430                1435                1440

Gly Glu Leu Glu Ile Gln Ala Thr Trp Tyr Glu Lys Leu His Glu Trp
             1445                1450                1455

Glu Asp Ala Leu Val Ala Tyr Asp Lys Lys Met Asp Thr Asn Lys Asp
        1460                1465                1470

Asp Pro Glu Leu Met Leu Gly Arg Met Arg Cys Leu Glu Ala Leu Gly
        1475                1480                1485

Glu Trp Gly Gln Leu His Gln Gln Cys Cys Glu Lys Trp Thr Leu Val
   1490                1495                1500

Asn Asp Glu Thr Gln Ala Lys Met Ala Arg Met Ala Ala Ala Ala Ala
1505                1510                1515                1520

Trp Gly Leu Gly Gln Trp Asp Ser Met Glu Glu Tyr Thr Cys Met Ile
             1525                1530                1535
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Pro Arg Asp Thr His Asp Gly Ala Phe Tyr Arg Ala Val Leu Ala Leu
        1540            1545                1550

His Gln Asp Leu Phe Ser Leu Ala Gln Gln Cys Ile Asp Lys Ala Arg
        1555            1560                1565

Asp Leu Leu Asp Ala Glu Leu Thr Ala Met Ala Gly Glu Ser Tyr Ser
    1570            1575                1580

Arg Ala Tyr Gly Ala Met Val Ser Cys His Met Leu Ser Glu Leu Glu
1585            1590                1595                1600

Glu Val Ile Gln Tyr Lys Leu Val Pro Glu Arg Arg Glu Ile Ile Arg
                1605            1610                1615

Gln Ile Trp Trp Glu Arg Leu Gln Gly Cys Gln Arg Ile Val Glu Asp
            1620            1625                1630

Trp Gln Lys Ile Leu Met Val Arg Ser Leu Val Val Ser Pro His Glu
        1635            1640                1645

Asp Met Arg Thr Trp Leu Lys Tyr Ala Ser Leu Cys Gly Lys Ser Gly
    1650            1655                1660

Arg Leu Ala Leu Ala His Lys Thr Leu Val Leu Leu Leu Gly Val Asp
1665            1670                1675                1680

Pro Ser Arg Gln Leu Asp His Pro Leu Pro Thr Val His Pro Gln Val
            1685            1690                1695

Thr Tyr Ala Tyr Met Lys Asn Met Trp Lys Ser Ala Arg Lys Ile Asp
            1700            1705                1710

Ala Phe Gln His Met Gln His Phe Val Gln Thr Met Gln Gln Gln Ala
        1715            1720                1725

Gln His Ala Ile Ala Thr Glu Asp Gln Gln His Lys Gln Glu Leu His
    1730            1735                1740

Lys Leu Met Ala Arg Cys Phe Leu Lys Leu Gly Glu Trp Gln Leu Asn
1745            1750                1755                1760

Leu Gln Gly Ile Asn Glu Ser Thr Ile Pro Lys Val Leu Gln Tyr Tyr
            1765            1770                1775

Ser Ala Ala Thr Glu His Asp Arg Ser Trp Tyr Lys Ala Trp His Ala
            1780            1785                1790

Trp Ala Val Met Asn Phe Glu Ala Val Leu His Tyr Lys His Gln Asn
        1795            1800                1805

Gln Ala Arg Asp Glu Lys Lys Lys Leu Arg His Ala Ser Gly Ala Asn
    1810            1815                1820

Ile Thr Asn Ala Thr Thr Thr Ala Thr Thr Ala Ala Ser Ala Ala Ala
1825            1830                1835                1840

Ala Thr Ser Thr Glu Gly Ser Asn Ser Glu Ser Glu Ala Glu Ser Asn
            1845            1850                1855

Glu Ser Ser Pro Thr Pro Ser Pro Leu Gln Lys Lys Val Thr Glu Asp
        1860            1865                1870

Leu Ser Lys Thr Leu Leu Leu Tyr Thr Val Pro Ala Val Gln Gly Phe
        1875            1880                1885

Phe Arg Ser Ile Ser Leu Ser Arg Gly Asn Asn Leu Gln Asp Thr Leu
    1890            1895                1900

Arg Val Leu Thr Leu Trp Phe Asp Tyr Gly His Trp Pro Asp Val Asn
1905            1910                1915                1920

Glu Ala Leu Val Glu Gly Val Lys Ala Ile Gln Ile Asp Thr Trp Leu
            1925            1930                1935

Gln Val Ile Pro Gln Leu Ile Ala Arg Ile Asp Thr Pro Arg Pro Leu
        1940            1945                1950

Val Gly Arg Leu Ile His Gln Leu Leu Thr Asp Ile Gly Arg Tyr His
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          1955              1960              1965

Pro Gln Ala Leu Ile Tyr Pro Leu Thr Val Ala Ser Lys Ser Thr Thr
    1970              1975              1980

Thr Ala Arg His Asn Ala Ala Asn Lys Ile Leu Lys Asn Met Cys Glu
1985              1990              1995              2000

His Ser Asn Thr Leu Val Gln Gln Ala Met Met Val Ser Glu Glu Leu
              2005              2010              2015

Ile Arg Val Ala Ile Leu Trp His Glu Met Trp His Glu Gly Leu Glu
              2020              2025              2030

Glu Ala Ser Arg Leu Tyr Phe Gly Glu Arg Asn Val Lys Gly Met Phe
              2035              2040              2045

Glu Val Leu Glu Pro Leu His Ala Met Met Glu Arg Gly Pro Gln Thr
    2050              2055              2060

Leu Lys Glu Thr Ser Phe Asn Gln Ala Tyr Gly Arg Asp Leu Met Glu
2065              2070              2075              2080

Ala Gln Glu Trp Cys Arg Lys Tyr Met Lys Ser Gly Asn Val Lys Asp
              2085              2090              2095

Leu Thr Gln Ala Trp Asp Leu Tyr Tyr His Val Phe Arg Arg Ile Ser
              2100              2105              2110

Lys Gln Leu Pro Gln Leu Thr Ser Leu Glu Leu Gln Tyr Val Ser Pro
    2115              2120              2125

Lys Leu Leu Met Cys Arg Asp Leu Glu Leu Ala Val Pro Gly Thr Tyr
    2130              2135              2140

Asp Pro Asn Gln Thr Ile Ile Arg Ile Gln Ser Ile Ala Pro Ser Leu
2145              2150              2155              2160

Gln Val Ile Thr Ser Lys Gln Arg Pro Arg Lys Leu Thr Leu Met Gly
              2165              2170              2175

Ser Asn Gly His Glu Phe Val Phe Leu Leu Lys Gly His Glu Asp Leu
              2180              2185              2190

Arg Gln Asp Glu Arg Val Met Gln Leu Phe Gly Leu Val Asn Thr Leu
    2195              2200              2205

Leu Ala Asn Asp Pro Thr Ser Leu Arg Lys Asn Leu Ser Ile Gln Arg
    2210              2215              2220

Tyr Ala Val Ile Pro Leu Ser Thr Asn Ser Gly Leu Ile Gly Trp Val
2225              2230              2235              2240

Pro His Cys Asp Thr Leu His Ala Leu Ile Arg Asp Tyr Arg Glu Lys
              2245              2250              2255

Lys Lys Ile Leu Leu Asn Ile Glu His Arg Ile Met Leu Arg Met Ala
              2260              2265              2270

Pro Asp Tyr Asp His Leu Thr Leu Met Gln Lys Val Glu Val Phe Glu
    2275              2280              2285

His Ala Val Asn Asn Thr Ala Gly Asp Asp Leu Ala Lys Leu Leu Trp
    2290              2295              2300

Leu Lys Ser Pro Ser Ser Glu Val Trp Phe Asp Arg Arg Thr Asn Tyr
2305              2310              2315              2320

Thr Arg Ser Leu Ala Val Met Ser Met Val Gly Tyr Ile Leu Gly Leu
              2325              2330              2335

Gly Asp Arg His Pro Ser Asn Leu Met Leu Asp Arg Leu Ser Gly Lys
              2340              2345              2350

Ile Leu His Ile Asp Phe Gly Asp Cys Phe Glu Val Ala Met Thr Arg
              2355              2360              2365

Glu Lys Phe Pro Glu Lys Ile Pro Phe Arg Leu Thr Arg Met Leu Thr
    2370              2375              2380
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```
Asn Ala Met Glu Val Thr Gly Leu Asp Arg Asn Tyr Arg Thr Thr Cys
2385                2390                2395                2400

His Thr Val Met Glu Val Leu Arg Glu His Lys Asp Ser Val Met Ala
            2405                2410                2415

Val Leu Glu Ala Phe Val Tyr Asp Pro Leu Leu Asn Trp Arg Leu Met
            2420                2425                2430

Asp Thr Asn Ala Lys Gly Asn Lys Arg Ser Arg Thr Arg Thr Asp Ser
            2435                2440                2445

Tyr Ser Ala Gly Gln Ser Val Glu Ile Leu Asp Gly Val Glu Leu Gly
        2450                2455                2460

Glu Pro Ala His Lys Lys Thr Gly Thr Thr Val Pro Glu Ser Ile His
2465                2470                2475                2480

Ser Phe Ile Gly Asp Gly Leu Val Lys Pro Glu Ala Leu Asn Lys Lys
                2485                2490                2495

Ala Ile Gln Ile Ile Asn Arg Val Arg Asp Lys Leu Thr Gly Arg Asp
            2500                2505                2510

Phe Ser His Asp Asp Thr Leu Asp Val Pro Thr Gln Val Glu Leu Leu
        2515                2520                2525

Ile Lys Gln Ala Thr Ser His Glu Asn Leu Cys Gln Cys Tyr Ile Gly
    2530                2535                2540

Trp Cys Pro Phe Trp
2545
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 2470 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Saccharomyces cerevisiae

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Pro His Glu Glu Gln Ile Trp Lys Ser Lys Leu Leu Lys Ala
1               5                   10                  15

Ala Asn Asn Asp Met Asp Met Asp Arg Asn Val Pro Leu Ala Pro Asn
            20                  25                  30

Leu Asn Val Asn Met Asn Met Lys Met Asn Ala Ser Arg Asn Gly Asp
        35                  40                  45

Glu Phe Gly Leu Thr Ser Ser Arg Phe Gly Gly Val Val Ile Gly Ser
    50                  55                  60

Asn Gly Asp Val Asn Phe Lys Pro Ile Leu Glu Lys Ile Phe Arg Glu
65                  70                  75                  80

Leu Thr Ser Asp Tyr Lys Glu Glu Arg Lys Leu Ala Ser Ile Ser Leu
                85                  90                  95

Phe Asp Leu Leu Val Ser Leu Glu His Glu Leu Ser Ile Glu Glu Phe
            100                 105                 110

Gln Ala Ile Ser Asn Asp Ile Asn Asn Lys Ile Leu Glu Leu Val His
        115                 120                 125

Thr Lys Lys Thr Asn Thr Arg Val Gly Ala Val Leu Ser Ile Asp Thr
    130                 135                 140

Leu Ile Ser Phe Tyr Ala Tyr Thr Glu Arg Leu Pro Asn Glu Thr Ser
145                 150                 155                 160
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Arg Leu Ala Gly Tyr Leu Arg Gly Leu Ile Pro Ser Asn Asp Val Glu
            165                 170                 175

Val Met Arg Leu Ala Ala Lys Thr Leu Gly Lys Leu Ala Val Pro Gly
            180                 185                 190

Gly Thr Tyr Thr Ser Asp Phe Val Glu Phe Glu Ile Lys Ser Cys Leu
            195                 200                 205

Glu Trp Leu Thr Ala Ser Thr Glu Lys Asn Ser Phe Ser Ser Ser Lys
        210                 215                 220

Pro Asp His Ala Lys His Ala Ala Leu Leu Ile Ile Thr Ala Leu Ala
225                 230                 235                 240

Glu Asn Cys Pro Tyr Leu Leu Tyr Gln Tyr Leu Asn Ser Ile Leu Asp
                245                 250                 255

Asn Ile Trp Arg Ala Leu Arg Asp Pro His Leu Val Ile Arg Ile Asp
                260                 265                 270

Ala Ser Ile Thr Leu Ala Lys Cys Leu Ser Thr Leu Arg Asn Arg Asp
            275                 280                 285

Pro Gln Leu Thr Ser Gln Trp Val Gln Arg Leu Ala Thr Ser Cys Glu
        290                 295                 300

Tyr Gly Phe Gln Val Asn Thr Leu Glu Cys Ile His Ala Ser Leu Leu
305                 310                 315                 320

Val Tyr Lys Glu Ile Leu Phe Leu Lys Asp Pro Phe Leu Asn Gln Val
                325                 330                 335

Phe Asp Gln Met Cys Leu Asn Cys Ile Ala Tyr Glu Asn His Lys Ala
            340                 345                 350

Lys Met Ile Arg Glu Lys Ile Tyr Gln Ile Val Pro Leu Leu Ala Ser
            355                 360                 365

Phe Asn Pro Gln Leu Phe Ala Gly Lys Tyr Leu His Gln Ile Met Asp
        370                 375                 380

Asn Tyr Leu Glu Ile Leu Thr Asn Ala Pro Ala Lys Lys Ile Pro His
385                 390                 395                 400

Leu Lys Asp Asp Lys Pro Gln Ile Leu Ile Ser Ile Gly Asp Ile Ala
                405                 410                 415

Tyr Glu Val Gly Pro Asp Ile Ala Pro Tyr Val Lys Gln Ile Leu Asp
            420                 425                 430

Tyr Ile Glu His Asp Leu Gln Thr Lys Phe Lys Phe Arg Lys Lys Phe
            435                 440                 445

Glu Asn Glu Ile Phe Tyr Cys Ile Gly Arg Leu Ala Val Pro Leu Gly
        450                 455                 460

Pro Val Leu Gly Lys Leu Leu Asn Arg Asn Ile Leu Asp Leu Met Phe
465                 470                 475                 480

Lys Cys Pro Leu Ser Asp Tyr Met Gln Glu Thr Phe Gln Ile Leu Thr
                485                 490                 495

Glu Arg Ile Pro Ser Leu Gly Pro Lys Ile Asn Asp Glu Leu Leu Asn
            500                 505                 510

Leu Val Cys Ser Thr Leu Ser Gly Thr Pro Phe Ile Gln Pro Gly Ser
            515                 520                 525

Pro Met Glu Ile Pro Ser Phe Ser Arg Glu Arg Ala Arg Glu Trp Arg
        530                 535                 540

Asn Lys Ser Ile Leu Gln Lys Thr Gly Glu Ser Asn Asp Asp Asn Asn
545                 550                 555                 560

Asp Ile Lys Ile Ile Ile Gln Ala Phe Arg Met Leu Lys Asn Ile Lys
                565                 570                 575

Ser Arg Phe Ser Leu Val Glu Phe Val Arg Ile Val Ala Leu Ser Tyr
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              580                    585                         590
Ile Glu His Thr Asp Pro Arg Val Arg Lys Leu Ala Ala Leu Thr Ser
        595                    600              605

Cys Glu Ile Tyr Val Lys Asp Asn Ile Cys Lys Gln Thr Ser Leu His
        610                    615              620

Ser Leu Asn Thr Val Ser Glu Val Leu Ser Lys Leu Leu Ala Ile Thr
625                    630              635                    640

Ile Ala Asp Pro Leu Gln Asp Ile Arg Leu Glu Val Leu Lys Asn Leu
                 645              650                    655

Asn Pro Cys Phe Asp Pro Gln Leu Ala Gln Pro Asp Asn Leu Arg Leu
             660              665              670

Leu Phe Thr Ala Leu His Asp Glu Ser Phe Asn Ile Gln Ser Val Ala
        675              680              685

Met Glu Leu Val Gly Arg Leu Ser Ser Val Asn Pro Ala Tyr Val Ile
        690              695              700

Pro Ser Ile Arg Lys Ile Leu Leu Glu Leu Leu Thr Lys Leu Lys Phe
705              710              715                    720

Ser Thr Ser Ser Arg Glu Lys Glu Glu Thr Ala Ser Leu Leu Cys Thr
                 725              730              735

Leu Ile Arg Ser Ser Lys Asp Val Ala Lys Pro Tyr Ile Glu Pro Leu
             740              745              750

Leu Asn Val Leu Leu Pro Lys Phe Gln Asp Thr Ser Ser Thr Val Ala
        755              760              765

Ser Thr Ala Leu Arg Thr Ile Gly Glu Leu Ser Val Val Gly Gly Glu
        770              775              780

Asp Met Lys Ile Tyr Leu Lys Asp Leu Phe Pro Leu Ile Ile Lys Thr
785              790              795                    800

Phe Gln Asp Gln Ser Asn Ser Phe Lys Arg Glu Ala Ala Leu Lys Ala
                 805              810              815

Leu Gly Gln Leu Ala Ala Ser Ser Gly Tyr Val Ile Asp Pro Leu Leu
             820              825              830

Asp Tyr Pro Glu Leu Leu Gly Ile Leu Val Asn Ile Leu Lys Thr Glu
        835              840              845

Asn Ser Gln Asn Ile Arg Arg Gln Thr Val Thr Leu Ile Gly Ile Leu
850              855              860

Gly Ala Ile Asp Pro Tyr Arg Gln Lys Glu Arg Glu Val Thr Ser Thr
865              870              875                    880

Thr Asp Ile Ser Thr Glu Gln Asn Ala Pro Pro Ile Asp Ile Ala Leu
                 885              890              895

Leu Met Gln Gly Met Ser Pro Ser Asn Asp Glu Tyr Tyr Thr Thr Val
        900              905              910

Val Ile His Cys Leu Leu Lys Ile Leu Lys Asp Pro Ser Leu Ser Ser
        915              920              925

Tyr His Thr Ala Val Ile Gln Ala Ile Met His Ile Phe Gln Thr Leu
        930              935              940

Gly Leu Lys Cys Val Ser Phe Leu Asp Gln Ile Ile Pro Thr Ile Leu
945              950              955                    960

Asp Val Met Arg Thr Cys Ser Gln Ser Leu Leu Glu Phe Tyr Phe Gln
                 965              970              975

Gln Leu Cys Ser Leu Ile Ile Ile Val Arg Gln His Ile Arg Pro His
             980              985              990

Val Asp Ser Ile Phe Gln Ala Ile Lys Asp Phe Ser Ser Val Ala Lys
        995              1000             1005
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Leu Gln Ile Thr Leu Val Ser Val Ile Glu Ala Ile Ser Lys Ala Leu
    1010            1015            1020

Glu Gly Glu Phe Lys Arg Leu Val Pro Leu Thr Leu Thr Leu Phe Leu
1025            1030            1035            1040

Val Ile Leu Glu Asn Asp Lys Ser Ser Asp Lys Val Leu Ser Arg Arg
            1045            1050            1055

Val Leu Arg Leu Leu Glu Ser Phe Gly Pro Asn Leu Glu Gly Tyr Ser
        1060            1065            1070

His Leu Ile Thr Pro Lys Ile Val Gln Met Ala Glu Phe Thr Ser Gly
    1075            1080            1085

Asn Leu Gln Arg Ser Ala Ile Ile Thr Ile Gly Lys Leu Ala Lys Asp
    1090            1095            1100

Val Asp Leu Phe Glu Met Ser Ser Arg Ile Val His Ser Leu Leu Arg
1105            1110            1115            1120

Val Leu Ser Ser Thr Thr Ser Asp Glu Leu Ser Lys Val Ile Met Asn
            1125            1130            1135

Thr Leu Ser Leu Leu Leu Ile Gln Met Gly Thr Ser Phe Ala Ile Phe
        1140            1145            1150

Ile Pro Val Ile Asn Glu Val Leu Met Lys Lys His Ile Gln His Thr
        1155            1160            1165

Ile Tyr Asp Asp Leu Thr Asn Arg Ile Leu Asn Asn Asp Val Leu Pro
    1170            1175            1180

Thr Lys Ile Leu Glu Ala Asn Thr Thr Asp Tyr Lys Pro Ala Glu Gln
1185            1190            1195            1200

Met Glu Ala Ala Asp Ala Gly Val Ala Lys Leu Pro Ile Asn Gln Ser
            1205            1210            1215

Val Leu Lys Ser Ala Trp Asn Ser Ser Gln Gln Arg Thr Lys Glu Asp
        1220            1225            1230

Trp Gln Glu Trp Ser Lys Arg Leu Ser Ile Gln Leu Leu Lys Glu Ser
    1235            1240            1245

Pro Ser His Ala Leu Arg Ala Cys Ser Asn Leu Ala Ser Met Tyr Tyr
    1250            1255            1260

Pro Leu Ala Lys Glu Leu Phe Asn Thr Ala Phe Ala Cys Val Trp Thr
1265            1270            1275            1280

Glu Leu Tyr Ser Gln Tyr Gln Glu Asp Leu Ile Gly Ser Leu Cys Ile
            1285            1290            1295

Ala Leu Ser Ser Pro Leu Asn Pro Pro Glu Ile His Gln Thr Leu Leu
        1300            1305            1310

Asn Leu Val Glu Phe Met Glu His Asp Asp Lys Ala Leu Pro Ile Pro
    1315            1320            1325

Thr Gln Ser Leu Gly Glu Tyr Ala Glu Arg Cys His Ala Tyr Ala Lys
    1330            1335            1340

Ala Leu His Tyr Lys Glu Ile Lys Phe Ile Lys Glu Pro Glu Asn Ser
1345            1350            1355            1360

Thr Ile Glu Ser Leu Ile Ser Ile Asn Asn Gln Leu Asn Gln Thr Asp
            1365            1370            1375

Ala Ala Ile Gly Ile Leu Lys His Ala Gln Gln His His Ser Leu Gln
        1380            1385            1390

Leu Lys Glu Thr Trp Phe Glu Lys Leu Glu Arg Trp Glu Asp Ala Leu
    1395            1400            1405

His Ala Tyr Asn Glu Arg Glu Lys Ala Gly Asp Thr Ser Val Ser Val
    1410            1415            1420
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Thr Leu Gly Lys Met Arg Ser Leu His Ala Leu Gly Glu Trp Glu Gln
1425                1430            1435                1440

Leu Ser Gln Leu Ala Ala Arg Lys Trp Lys Val Ser Lys Leu Gln Thr
                1445            1450                1455

Lys Lys Leu Ile Ala Pro Leu Ala Ala Gly Ala Arg Trp Gly Leu Gly
                1460            1465                1470

Glu Trp Asp Met Leu Glu Gln Tyr Ile Ser Val Met Lys Pro Lys Ser
                1475            1480            1485

Pro Asp Lys Glu Phe Phe Asp Ala Ile Leu Tyr Leu His Lys Asn Asp
        1490            1495            1500

Tyr Asp Asn Ala Ser Lys His Ile Leu Asn Ala Arg Asp Leu Leu Val
1505                1510            1515                1520

Thr Glu Ile Ser Ala Leu Ile Asn Glu Ser Tyr Asn Arg Ala Tyr Ser
                1525            1530                1535

Val Ile Val Arg Thr Gln Ile Ile Thr Glu Phe Glu Glu Ile Ile Lys
                1540            1545                1550

Tyr Lys Gln Leu Pro Pro Asn Ser Glu Lys Lys Leu His Tyr Gln Asn
        1555            1560            1565

Leu Trp Thr Lys Arg Leu Leu Gly Cys Gln Lys Asn Val Asp Leu Trp
        1570            1575            1580

Gln Arg Val Leu Arg Val Arg Ser Leu Val Ile Lys Pro Lys Gln Asp
1585                1590            1595                1600

Leu Gln Ile Trp Ile Lys Phe Ala Asn Leu Cys Arg Lys Ser Gly Arg
                1605            1610                1615

Met Arg Leu Ala Asn Lys Ala Leu Asn Met Leu Leu Glu Gly Gly Asn
                1620            1625            1630

Asp Pro Ser Leu Pro Asn Thr Val Lys Ala Pro Pro Val Val Tyr
        1635            1640            1645

Ala Gln Leu Lys Tyr Ile Trp Ala Thr Gly Ala Tyr Lys Glu Ala Leu
        1650            1655            1660

Asn His Leu Ile Gly Phe Thr Ser Arg Leu Ala His Asp Leu Gly Leu
1665                1670            1675                1680

Asp Pro Asn Asn Met Ile Ala Gln Ser Val Lys Leu Ser Ser Ala Ser
                1685            1690            1695

Thr Ala Pro Tyr Val Glu Glu Tyr Thr Lys Leu Leu Ala Arg Cys Phe
                1700            1705            1710

Leu Lys Gln Gly Glu Trp Arg Ile Ala Thr Gln Pro Asn Trp Arg Asn
        1715            1720            1725

Thr Asn Pro Asp Ala Ile Leu Gly Ser Tyr Leu Leu Ala Thr His Phe
        1730            1735            1740

Asp Lys Asn Trp Tyr Lys Ala Trp His Asn Trp Ala Leu Ala Asn Phe
1745                1750            1755                1760

Glu Val Ile Ser Met Val Gln Glu Glu Thr Lys Leu Asn Gly Gly Lys
                1765            1770                1775

Asn Asp Asp Asp Asp Asp Thr Ala Val Asn Asn Asp Asn Val Arg Ile
                1780            1785            1790

Asp Gly Ser Ile Leu Gly Ser Gly Ser Leu Thr Ile Asn Gly Asn Arg
        1795            1800            1805

Tyr Pro Leu Glu Leu Ile Gln Arg His Val Val Pro Ala Ile Lys Gly
        1810            1815            1820

Phe Phe His Ser Ile Ser Leu Leu Glu Thr Ser Cys Leu Gln Asp Thr
1825                1830            1835                1840

Leu Arg Leu Leu Thr Leu Leu Phe Asn Phe Gly Gly Ile Lys Glu Val
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                1845              1850              1855

Ser Gln Ala Met Tyr Glu Gly Phe Asn Leu Met Lys Ile Glu Asn Trp
        1860              1865              1870

Leu Glu Val Leu Pro Gln Leu Ile Ser Arg Ile His Gln Pro Asp Pro
        1875              1880              1885

Thr Val Ser Asn Ser Leu Leu Ser Leu Leu Ser Asp Leu Gly Lys Ala
    1890              1895              1900

His Pro Gln Ala Leu Val Tyr Pro Leu Thr Val Ala Ile Lys Ser Glu
1905              1910              1915              1920

Ser Val Ser Arg Gln Lys Ala Ala Leu Ser Ile Ile Glu Lys Ile Arg
            1925              1930              1935

Ile His Ser Pro Val Leu Val Asn Gln Ala Glu Leu Val Ser His Glu
            1940              1945              1950

Leu Ile Arg Val Ala Val Leu Trp His Glu Leu Trp Tyr Glu Gly Leu
        1955              1960              1965

Glu Asp Ala Arg Arg Gln Phe Phe Val Glu His Asn Ile Glu Lys Met
    1970              1975              1980

Phe Ser Thr Leu Glu Pro Leu His Lys His Leu Gly Asn Glu Pro Gln
1985              1990              1995              2000

Thr Leu Ser Glu Val Ser Phe Gln Lys Ser Phe Gly Arg Asp Leu Asn
            2005              2010              2015

Asp Ala Tyr Glu Trp Leu Asn Asn Tyr Lys Lys Ser Lys Asp Ile Asn
            2020              2025              2030

Asn Leu Asn Gln Ala Trp Asp Ile Tyr Tyr Asn Val Phe Arg Lys Ile
        2035              2040              2045

Thr Arg Gln Ile Pro Gln Leu Gln Thr Leu Asp Leu Gln His Val Ser
    2050              2055              2060

Pro Gln Leu Leu Ala Thr His Asp Leu Glu Leu Ala Val Pro Gly Thr
2065              2070              2075              2080

Tyr Phe Pro Gly Lys Pro Thr Ile Arg Ile Ala Lys Phe Glu Pro Leu
            2085              2090              2095

Phe Ser Val Ile Ser Ser Lys Gln Arg Pro Arg Lys Phe Ser Ile Lys
        2100              2105              2110

Gly Ser Asp Gly Lys Asp Tyr Lys Tyr Val Leu Lys Gly His Glu Asp
        2115              2120              2125

Ile Arg Gln Asp Ser Leu Val Met Gln Leu Phe Gly Leu Val Asn Thr
    2130              2135              2140

Leu Leu Lys Asn Asp Ser Glu Cys Phe Lys Arg His Leu Asp Ile Gln
2145              2150              2155              2160

Gln Tyr Pro Ala Ile Pro Leu Ser Pro Lys Ser Gly Leu Leu Gly Trp
            2165              2170              2175

Val Pro Asn Ser Asp Thr Phe His Val Leu Ile Arg Glu His Arg Asp
        2180              2185              2190

Ala Lys Lys Ile Pro Leu Asn Ile Glu Gln Trp Val Met Leu Gln Met
        2195              2200              2205

Ala Pro Asp Tyr Glu Asn Leu Thr Leu Leu Gln Lys Ile Glu Val Phe
    2210              2215              2220

Thr Tyr Ala Leu Asp Asn Thr Lys Gly Gln Asp Leu Tyr Lys Ile Leu
2225              2230              2235              2240

Trp Leu Lys Ser Arg Ser Ser Glu Thr Trp Leu Glu Arg Arg Thr Thr
            2245              2250              2255

Tyr Thr Arg Ser Leu Ala Val Met Ser Met Thr Gly Tyr Ile Leu Gly
        2260              2265              2270
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